GENETIC ASSOCIATION STUDIES OF ASTHMA IN PAKISTANI POPULATION

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Pakistan

2014

GENETIC ASSOCIATION STUDIES OF ASTHMA IN PAKISTANI POPULATION

by

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97-Arid-329

A thesis submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

in

Biochemistry

Department of Biochemistry

Faculty of Sciences

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Pakistan

2014

CERTIFICATION

I hereby undertake that this research is an original one and no part of this thesis falls under plagiarism. If found otherwise, at any stage, I will be responsible for the consequences.

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Dedicated

To

My Loving Parents

Mr. and Mrs. Mahboob Sultan (late)

Whose best wishes, prayers and guidance at every step made

me achieved such a success in life

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LIST OF ABBREVIATIONS

IgE	Immunoglobulin E
20p	Short arm of chromosome 20
5q	long arm of Chromosome 5
ADAM	A Disintegrin and Metalloprotease
BHR	Bronchial hyper responsiveness
bp	Base pair
CD	Cluster of Differentiation
dNTP	Deoxy-Nucleotide Triphosphate
EDTA	Ethylenediamine Tetra Acetic Acid
IL	Interleukin
kDa	Kilo Dalton
kg	Kilo gram
M	Molar
MAF	Minor Allele Frequency
min	Minutes
mL	Milliliter
mM	Milli molar
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide

NH_4	Ammonia
nm	Nanometer
OPD	Out Patient Department
PCR	Polymerase chain reaction
RBCs	Red Blood Cells
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolution Per Minute
rs	SNP reference
SDS	Sodium Dodecyl Sulphate
sec	Seconds
SNP	Single Nucleotide Polymorphism
SPSS	Statistical Package for Social Sciences
TAE	Tris Acetate EDTA
TE	Tris-EDTA
TGF	Tumor Growth Factor
Th2	T-helper cells
TNF	Tumor Necrosis factor
U	Units
V	Volts
Zn	Zinc
α	Alpha

β	Beta
µg	Microgram
µL	Micro liter

ACKNOWLEDGEMENTS

I bow my head to **Allah**, the Almighty, the compassionate, most beneficent, most merciful, who blessed me good health, very kind parents, talented teachers and a conductive environment that ultimately resulted in completing this work. All praises and respects are for **Holy Prophet Muhammad (PBUH)**, the fortune of knowledge, who took the humanity out of the abyss of ignorance and elevated it to the zenith of consciousness and enabled us to recognize our creator.

I would like to show my cordial gratitude to my respected supervisor **Dr. Ghazala Kaukab Raja**, Associate Professor, Department of Biochemistry, PMAS-AAUR who took me as her Ph.D. student and provided me an opportunity to work with her at **Institute of Biomedical and Genetic Engineering (IB&GE), KRL.** I would like to thank **Dr. Muhammad Ismail** Director General **IB&GE**, who helped me to work under the guidance of highly professional personas in KRL**.** I am honored to express my deep sense of gratitude and indebtedness to my Co-supervisor **Dr. Atika Mansoor,** CSO in **IB&GE** who guided me at every step of my research. It was because of her inspiring guidance, encouragement and friendly attitude during the entire study program that I could prepare this manuscript. I am extremely grateful to her for giving me her precious time whenever I needed. I like

to thank **HEC** for giving me **IRSIP** scholarship to work at University of Chicago USA for completion of my research.

I owe many thanks and appreciation to **Dr. Sadia Rehman** (PSO), **Ms. Saeeda Munir (**SSO)**, Dr. Nafees Ahmad (**SSO) and **Dr. Rashda Abbasi (**SSO) at Institute of Biomedical and Genetic Engineering (**IB&GE)**, Islamabad for their kind, caring attitude, valuable suggestions and encouragement throughout the study, they have made available their support in a number of ways to carry out the research work smoothly which would not have been possible without their support and guidance.

I would like to thanks **Prof. Dr. Muhammad Gulfraz Satti**, Chairman, Department of Biochemistry, my Supervisory Committee members **Dr. Muhammad Sheeraz Ahmed** Lecturer, Department of Biochemistry, PMAS-AAUR and **Dr. Mazhar Qayyum**, Assistant Professor, Department of Biology/Zoology, PMAS-AAUR. I would like to extend my thanks to **Dr. Pakeeza Aarzoo Shaiq** Lecturer, Department of Biochemistry, PMAS-AAUR ,who helped me in write up of thesis and guided me in every possible way to her level best.

I would like to express my heartiest gratitude and thanks to my lab fellows at Institute of Biomedical and Genetic Engineering, Islamabad; and PMAS-Arid Agriculture University, Rawalpindi, especially to **Mr. M. Saqlain** (PhD Scholar),

Department of Biochemistry for helping me a lot in completion of this manuscript, **Mr. Abid Mehmood** (PhD Scholar), Department of Biochemistry PMAS-AAUR and all **my class fellows** for their help and support throughout my study period in university.

It would not have been possible to write this manuscript without help of my husband **Mr. Muhammad Naeem** and my children **Mohib-ur-Rehman**, **Muhammad Aayan** and **Anabia Noor** who suffer a lot during my whole course of thesis. I am thankful to them for their help, entertainment and care they provided me.

Words cannot express my profound admiration, devotion and gratitude to my affectionate late **parents** for their prayers during all my difficulties, and to my father in law **Mr. Ghulam Murtaza** for his prayers during all this.I am also very grateful to my brother**, Zia-ul-Haq** who supports me in every possible way and **Bhabi farah and children Eesha, Aashir, Shamir and Saim** for their constant support and motivation during this study period. Without the prayers and support of my late parents I could not have achieved this goal.

May **ALLAH ALMIGHTY** bless all of them at every stage of their lives, Ameen.

NUSRAT SABA

ABSTRACT

Asthma is an inflammatory chronic disorder of airways of lungs. In world, asthma is recognized as one of the most chronic diseases, which affects above 300 million people all round the world. In Pakistan, approximately 5-8% of adults are suffering from asthma. Both environmental and genetic factors contribute towards the development of disease. It is a multigenic disorder, and is due to different genetic factors, which also determine immune responses. The purpose of the present study is to identify and investigate polymorphisms in major asthma genes. In present study, 34 SNPs of 28 genes were genotyped by iPLEX and Taqman assay. HLA class II association was done by sequence specific PCR. It is found that the minor alleles of *IL10* (rs1800896) and *IL13* (rs1800925) are related to increased asthma risk in our population. It is also seen that the minor alleles of *ADAM33* (rs2280091) are showing significant association with protection in Pakistani asthmatic population. *ADAM33* minor allele is having protective effect in females. *ACE* homozygous insertion is reported to be associated with asthma risk in Pakistani population. HLA class II *DRB1*07* and *DQB1*03* are found to be important in asthma pathogenesis and *DQB1*06* is showing statistically significant association with protection against the disease.

Chapter 1

INTRODUCTION

Asthma is an inflammatory chronic disorder of airways of lungs. Many cells and mediators involved in asthma pathogenesis are eosinophils, mast cells and T-lymphocytes. Asthma could be environmentally triggered and or genetically inherited (Martinez 2007). Chronic inflammation is connected with bronchial hyperesponsivness and leads to episodes of wheezing, coughing, tightness in the chest, breathlessness, shortage of breath especially at night and in the morning. This episode is usually connected with variable obstruction, which is reversible spontaneously or by treatment.

Asthma is recognized as one of the most chronic diseases, which affect more than 300 million people all around the world and annually causing approximately 255,000 premature deaths (accounting for about one out of 250 deaths). It has been described that if necessary action is not taken, asthma deaths would increase by almost 20% in the coming ten years. In almost all countries, asthma occurs with variations in the level of development, but more than 80% deaths due to asthma occur in low and middle-income countries (Masoli *et al.*, 2004). In Pakistan, there is a day-by-day increase in asthma prevalence with an estimated 5% increase annually, out of which children between the age of 13 and 15 years are 20%.

Approximately 5-8% of adult Pakistani population is already suffering from the disease. Recent data compiled about the prevalence of asthma in Pakistan shows 19 per cent prevalence in children and five per cent in adults (Pak. Press Release, Feb 21st, 2009).

Asthma is the disease that is associated with allergy -atopic disease, in which deviation of the immune system is the reason due to which an inflammation of the airways of lungs occurs that is non infectious. Both environmental and genetic factors contribute towards the development of disease. Asthma is a multigenic disorder, and its development is due to different genetic factors, which also determine immune responses (Martinez 2007).

Asthma could run strongly in families and not only environment along with genetic factors interact in the asthma development but gene to gene interactions are also a major contributor. Immune system linked genes e.g., human leukocyte antigen (HLA) class II genes, cytokines, β-adrenergic receptor gene, angiotensin converting enzyme (ACE) are reported in asthma pathogenesis (Malerba and Pignatti, 2005). Highly polymorphic HLA class II genes, especially HLA-DQ genes, involved in antigenic presentation and T-cell restriction have been thought to be candidate loci for the etiology of asthma.

Different cytokines have also been associated with asthma. The pathway of Interleukin 4/ interleukin 13 is one of the most important signaling pathways in atopy. This has long been known from immunology that the important cytokine *IL-4* is involved in immunoglobulin E (IgE) switching in B-cells. In all atopic diseases, this common consequence is found. In recent studies, it is reported that *IL-13* is also a good mediator for immune responses in atopic diseases for the asthma induction as compared to *IL-4*. The T regulatory cell produces both of these interleukins – *IL-4* and *IL-13*. A common pathway having a shared receptor chain (*IL4Ra*) and an intracellular transducer for signal (*STAT6*) is involved in signaling of both cytokines (Ober and Hoffjan, 2006). In a meta-analysis study, *IL4RA* polymorphisms were reported in atopic asthma association (Nie *et al*, 2013).

Two genes *IL33* and IL-1 receptor-like 1 (*IL1RL1*) act in single signal transduction pathway. The cytokine released on damaging of cells is encoded by *IL33*, however part of the *IL-33* receptor complex is encoded by *IL1RL1*. Asthma is associated with genetic variation at the *IL33* and *IL1RL1* loci, which can be dissected into independent signals with distinct functional consequences central for pathway to pathogenesis of asthma (Grotenboer *et al.*, 2013). In immune tolerance, *IL-10* has an important role. An increased asthma risk and Th2-type immunology

has been associated with lower production of *IL-10*. *IL-10* was associated with childhood asthma in other world populations (Koponen *et al.,* 2013).

The expression of *IL-18* is very strong in the lungs in the fatal asthma group. In severe asthma patient's treatment, the *IL-18* signal transduction pathway may be of clinical benefit (Oda *et al.,* 2014). It has been concluded based on meta-analysis of results that *IL-4RA* Q576R polymorphism is significantly important for asthma risk (Xue *et al.,* 2010). In pathogenesis of atopic diseases including bronchial asthma, the involvement of *FcεRI* in the allergen-induced immune response is reported. *FcεRI* consists of 3 subunits, namely, α, ß, and γ chains. The β chain plays an important role in IgE-mediated allergic reactions (Akizawa *et al.,* 2003). In some of the studies, the association of *FCER1B* gene has been reported (Shirakawa *et al.,* 1996; Cui *et al.,* 2003; Dmitrieva-Zdorova *et al.,* 2012).

In addition to cytokines, some chemokines (a family of small,8 – 14 kDa secreted proteins whose major function is to guide the migration and development of leukocytes) have also been found playing some major role in inflammation (Charo and Ransohoff, 2006, Rot and Andrian, 2004). Due to their involvement in immune cell trafficking, chemokines have been implicated in a variety of inflammatory diseases, including rheumatoid arthritis, heart disease, asthma, type II diabetes and cancer (Acker *et al.,* 1996; Pickup 2004).

Besides cytokines, genetic variations in different loci and genes have also been reported. One such variation is at 17q21 locus, which is strongly associated with childhood nonallergic asthma. Expression of the 17q21 genes, orosomucoid (yeast) like protein isoform 3 (*ORMDL3*) and gasdermin B (*GSMDB*), is affected by different disease-associated variants (Ono *et al.,* 2014). *ORMDL3* has been identified as a candidate gene for susceptibility to asthma and its mechanisms by which it contributes to asthma pathogenesis is also reported in the context of allergic inflammation (Ha *et al.,* 2013). Some of the SNPs of *ORMDL3* have shown associations with asthma in several populations (Balantic *et al.,* 2013).

Two distinct genes *NOS1* and *NOS3* are present on chromosome 12 and 7 respectively. Human airway epithelial cells express these two *NOS* isoforms (Asano *et al.,* 1994). It is reported that *NOS1* and *NOS3* control levels of NO to perform physiological functions properly (Bouzigon *et al.,* 2012). The Neuropeptide S Receptor (*NPSR1*) gene on chromosome 7 has been associated to asthma in several populations (Castro-Giner *et al.,* 2010; Deley *et al.,* 2009). In some of the recent studies, *NPSR1* genotypes are also positively affecting the allergic diseases (Kauppi *et al.,* 2014).

Transcriptional activity in the *TBXA2R* gene was also influenced and identified in some genetic variants related to asthma phenotypes. In asthmatic cases thromboxane pathways have important roles in airway inflammation and remodelling (Takeuchi *et al.,* 2013). In several genetic studies gene involved in chronic airway inflammation, the gene coding thromboxane A2 receptor has been reported to be associated with asthma (Kavalar *et al.,* 2012). Transforming growth factor-β1 (TGF-β1) is one of several candidate loci for the pathogenesis of asthma, and is highly polymorphic (Che *et al.,* 2014). Similarly, airway inflammation and immune response, both characteristics seen in asthma are affected by tumor necrosis factor alpha (*TNFα*) (Witte *et al.,* 2002). TNFα levels may have impact from genetic factors, and some of the TNF gene polymorphisms, present on chromosome 6p21, have been found associated with TNFα production and potential increased risk of asthma. The TNF-alpha variant G-308A is studied in different populations and reported its association with asthma.

A member of the ADAM (a disintegrin and metalloprotease domain) family is ADAM 33. It is implicated in asthma and bronchial hyperresponsiveness and is a type I transmembrane protein. Its locus on chromosome 20p13 is identified on genome-wide screen of 460 Caucasian families. Its strong association with asthma was reported in world populations as *ADAM33* is expressed in lung (bronchial smooth muscle and fibroblasts) and lymph nodes. *ADAM33* is an excellent candidate gene for asthma susceptibility (Bouzigon *et al.,* 2008). Polymorphisms

in β-adrenergic receptor and *ACE* genes may also predict the response to asthma therapy. The *ACE* genes have been found important in pathogenesis of asthma. A variant of *ACE* is insertion/deletion (I/D) polymorphism, which is found in different populations (Eryüksel *et al.,* 2009). An important factor studied in asthma-related research is the beta-2-adrenergic receptor, which is encoded by the *ADRB2* gene (Thakkinstian *et al.,* 2006). The *ADRB2* gene is a small gene on chromosome 5q31-q32 (www.ncbi.nlm.nih.gov), a region genetically linked to asthma (Hawkins *et al.,* 2008). An interesting positive association between the responsiveness of asthmatic patients to β-adrenergic agonists and common arginine-16 variants in the β-adrenergic receptor gene is also reported (de Paiva *et al.,* 2014). Toll like receptor 4(*TLR4)* is also reported to be associated with allergic diseases (Ullah *et al.,* 2014).

There is much importance of *IL-4* and *IL-13* pathway in the IgE secretion regulation. Alterations in any main part of the described pathway can increase the risk of asthma development (Karp and Ewart, 2004). Different genes have been identified associated with asthma in worldwide genetic studies. Disease associated genotypes are taken as the same terms as other epidemiological risk factors which may be the predictor of disease and the size and relevance of effects of these genotypes can be judged objectively. Genes involved in asthma in Pakistani population have not been studied yet so is disease severity with different

environmental risk factors. It is hypothesized that Pakistani population might have different genetic make up from other world, which leads to asthma development. Therefore, the purpose of present study is to genotype Pakistani population for major asthma related genes under following objectives;

- Identification of asthma susceptibility genes in Pakistani population
- Investigation of polymorphisms in major asthma genes with asthma in Pakistani population

Chapter 2

REVIEW OF LITERATURE

Asthma is a respiratory disorder in humans, which is complex, and affects the airways that deliver air to the lungs. Its association is with narrowness of airways and leads to periodic attacks of wheezing, chest congestion, cough, and other similar symptoms resulting in difficulty in breathing. The immune system is triggered by asthma attacks leading to the elevated serum IgE levels. Along with various environmental factors, different genes are reported to be associated with an individual's ethnicity, susceptibility and asthma disease prevalence pattern. For the screening of individuals at risk, the identified genes and their associated variants are of considerable use. A number of genes and associated variants are reported to be linked strongly with increased asthma susceptibility in different ethnic groups all around the world. These genes are not screened in Pakistani population.

2.1 ASTHMA PREVALENCE

Regardless of the world development, asthma is rapidly increasing over the last 20 years all over the world. Approximately more than 5 million people in UK receive treatment for asthma, among them children are about 1 million and adults are more than 4 million. Bad management of asthma results in increased incidence of asthma and hospitalization of the patients due o asthma (Kaufman, 2011). In the

whole world, approximately 300 million people are suffering from asthma and there is a day by day increase in number of affected individuals. The asthma prevalence in our Pakistani population is increasing at the rate of 5% per year; among them children between 13 to 15 years of age are 20% to 30%. About 12% of the total Pakistani population, which is approximately 20 million, is affected with asthma (Pak, Press Release, May 2011). Since last two decades, there are marked established international guidelines for asthma treatment and management, still to cure the disease completely is a challenge. Even in developed countries such as UK there is high death rate that is three persons per day due to asthma, (Kaufman, 2011).

2.2 ASTHMA TYPES

Asthma is also sometimes known as bronchial asthma because the narrowing of the bronchial airways of lungs is involved in it. Asthma can be grouped as atopic which starts in childhood and is associated with risk factors which are identifiable and has genetic predisposition. Mostly asthma has an association with family history of allergic diseases. Allergic asthma is the one, which is increased by allergens, such as dander of pet, pollen, and preservatives of food or some fungus. Allergic asthma looks as seasonal because it is often due to allergies that are mostly seasonal. During asthma attack excessive IgE antibodies are produced from B lymphocytes which bind to inflammatory cells and result in narrowing of airways of

lungs making the breathe difficult. Every asthmatic case is not due to atopy. The patients who develop asthma at adult age may have viral respiratory infections as a secondary response and it is termed as non-atopic type of asthma. In this type of asthma immunoglobulin E (IgE) is not released and some other triggers are involved (Ward *et al.,* 2010).

2.3 ASTHMA PATHOPHYSIOLOGY

Asthma is triggered by various environmental allergens that lead to elevation of immune system. Different works have reported these environmental allergens, which include proteases, dust mites, pollens and animal dander. In the disease state an elevated expression of different cell types in bronchial mucosa such as, eosinophils, mast cells, macrophage, lymphocytes and basophils is seen. With these cells other inflammatory cells are involved in the airway inflammation which leads to the airways hyper responsiveness, limitation in airflow, symptoms of respiration, and chronic disease. Along with respiratory track cells, these cells bring about the changes in the structure of airways and increase resistance in airflow. Hereditary factors causing asthma are mostly affected by factors of environment. Various studies recommend that tissues microenvironment play a vital and major role at start, complexity and progression of asthma (Amrendra Kumar and Balaram Ghosh, 2009).

2.3.1 Effect on Lungs Airways

Inflammation in the walls of the airways during an asthma attack leads to swelling along with fluid accumulation in the walls covering the mucous membrane with fluid. The remaining space in the airways is filled by sticky mucus making it difficult to breathe. Because free movement of flow of air inside and outside of the lungs is not possible, a wheezing or whistling sound may be produced. During most damaging attacks there is no wheezing, the reason is that the amount of air that moves in to the air ways is too small to make such sound. These changes stimulate sensory nerves in the airways (Kaufman, 2011). Additionally when the surrounding muscles of the lungs tighten or go into spasm, the airways of the lungs may become blocked or obstructed. This retards air to move freely inside the lungs or the airways of the lungs may be clogged due to the mucus, resulting in difficulty to breathe.

Attacks of asthma may be mild, moderate, or harsh, and can last for a few seconds, minutes, hours, or few days. They can have occurrence anytime at any place. Mostly it occurs at the time of night. Sometimes, the signs and symptoms

are shown before an attack is going to occur, but sometimes there are no signs and symptoms before the attack.

2.3.2 Remodeling of Airways

Irreversible structural modifications in airways are often seen in chronic asthma due to abnormal remodeling and repair processes. Remodeling of airway is associated with epithelial damage, increased matrix deposition and smooth muscle hyperplasia. These changes in structure are mainly associated with the symptoms of asthma (Kedda *et al.,* 2006).

2.4 ASTHMA RISK AND TRIGGER FACTORS

As asthma is an interactive disease, the risk factors of asthma can be classified as environmental and genetic factors.

2.4.1 Environmental Factors

Many environmental risk factors are involved in asthma due to reason that the expression of disease susceptibility genesis increased. Allergens which are indoor such as fungus, cockroaches mites, dust in house, hair of animals are the environmental risk factors mainly associated with asthma. Allergens outside the

house include pollution in air, pollen, certain food items, active and passive smoke and clothing's. Certain drugs, obesity, infections in respiratory tract and parasitic viral or bacterial infections, for example, Chlamydia, *M. pneumonia* and mold are also included in risk factors. Inhalation of heavy smoke, ozone and endotoxin, as well as gastroesophageal backflow and emotional changes also induce nonallergic asthma (Abdulbari *et al.,* 2005). The severity of asthma attack is also affected by seasonal fluctuations. Smoking habits of mothers during pregnancy and atmospheric pollution can also result in elevated levels of IgE and asthma development and airway hyper responsivenessof lungs (Ward *et al.,* 2010).

2.4.2 Genetic Factors

The genetic factors include different genes present in the host that could be associated to different allergens somehow. The genetic factors contribute up to 79% alone (Holgate *et al.,* 2006).The literature emerging picture suggests asthma or asthma related phenotypes are associated with hundreds of genes as shown in figure 1 (Ober and Hoffjan, 2006; Zhang *et al.,* 2009). Currently major efforts are underway, taking use of new techniques such as genome-wide association studies of single-nucleotide-polymorphisms (SNP), to see importance of these genes in larger populations and also to identify novel and new genes (Moffatt *et al.,*2007; Ober *et al.,* 2008; Weidinger *et al.,* 2008).

More than 100 genes have been seen to be associated with asthma in at least one study regarding genetic association. However, these types of studies need to be replicated to ensure that the results are accurate. Towards the start of 2006, about 25 genes have been found to be associated with asthma in six or more different ethnic populations.

In at least 1,000 published papers, SNPs in genes that are candidates for asthma and allergy were examined. Although most of these genes have not been replicated, this is required to check whether these finding are true observations (Figure 2). The genes that are reported in many studies includ *ADAM33*, *FCER1B*,*ADRB2*, *IL4*, *CD14*, *HLA-DRB1*, *HLA-DQB1*, *TNF*, *IL4RA*, and *IL13* (Postma *et al.*, 2011). Immune system linked genes e.g., human leukocyte antigen (HLA) class II genes, cytokines, β-adrenergic receptor gene, angiotensin converting enzyme (ACE) are reported in asthma pathogenesis. Highly polymorphic HLA class II genes, especially HLA-DQ genes, involved in antigenic presentation and T-cell restriction have been thought to be candidate loci for the etiology of asthma (Malerba and Pignatti, 2005).

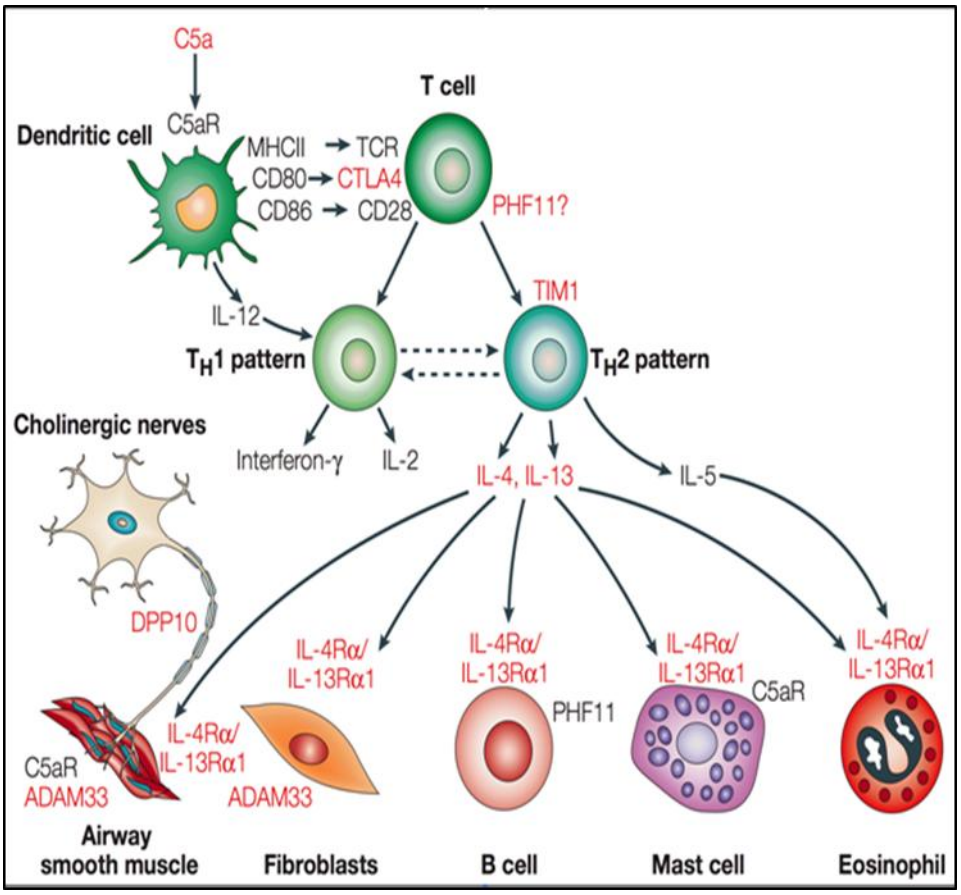

Figure 1: Hypothetical scheme of asthma pathogenesis. (Karp and Ewart, 2004).

Different cells are shown to be effected by various genes and then resulting in asthmatic pathogenesis

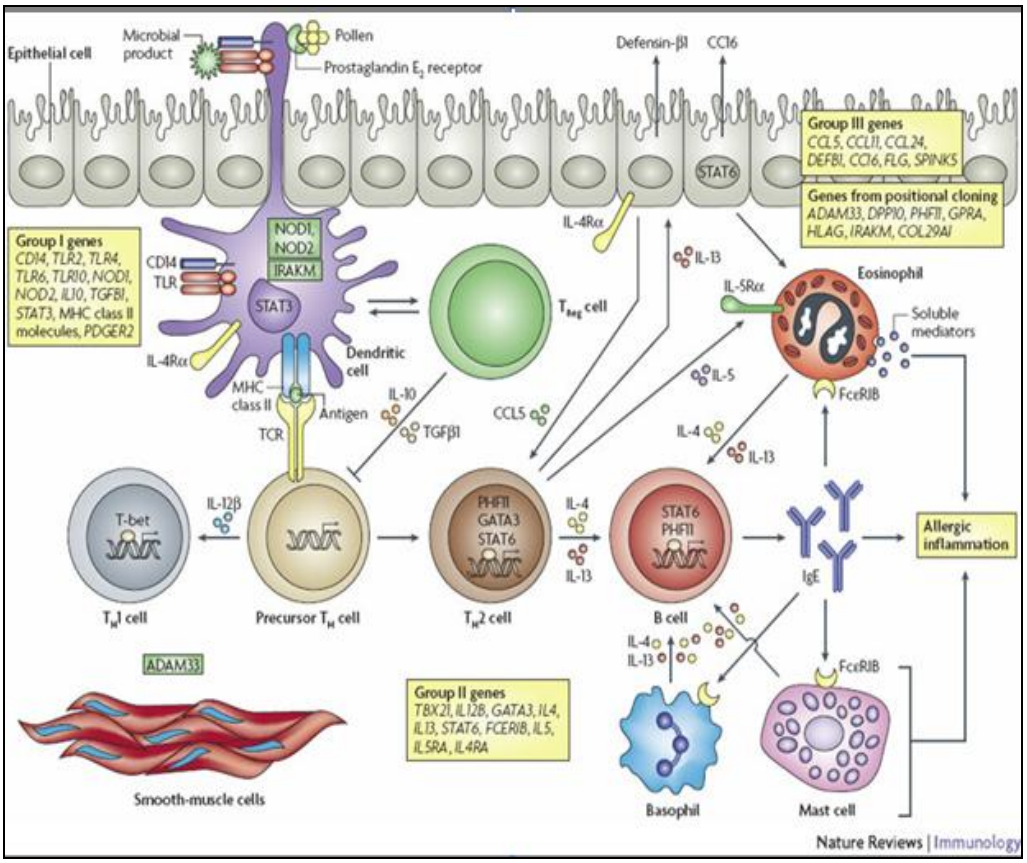

Figure 2: Susceptibility genes for asthma (Donata Vercelli 2008).

Pollens and allergens are effecting on dendritic cells and resulting in inflammation. This inflammation is effecting different group of genes at different levels and resulting in asthma pathogenesis.

In the present study, different genes and polymorphisms which have been found to be strongly associated with asthma in other populations were selected and from these the SNPs which have been reported in maximum studies were analyzed. The genes that were selected for analysis in the present study are described on the following pages.

2.5 HUMAN LEUKOCYTE ANTIGENS (HLA)

In humans, the major histocompatibility complex (MHC) is known as human leukocyte antigen (*HLA*) system. Multiple numbers of genes are present in this super locus which has relationship with function of immune system in humans. This is the gene group localized on chromosome 6, which encodes proteins presented on cell-surface antigens and several other genes.

The antigens of HLA class II (DM, DP, DOA, DOB, DQ, & DR) present antigens to T-lymphocytes from the outer side of cell. Multiplication of these T-helper cells is stimulated due to these specific antigens, and these T-helper cells stimulate antibody-producing B-cells to make antibodies in response to that specific antigen. Suppressor T-cells suppress these self-antigens. List of alleles of HLA DRB and DQB are shown in table 1.

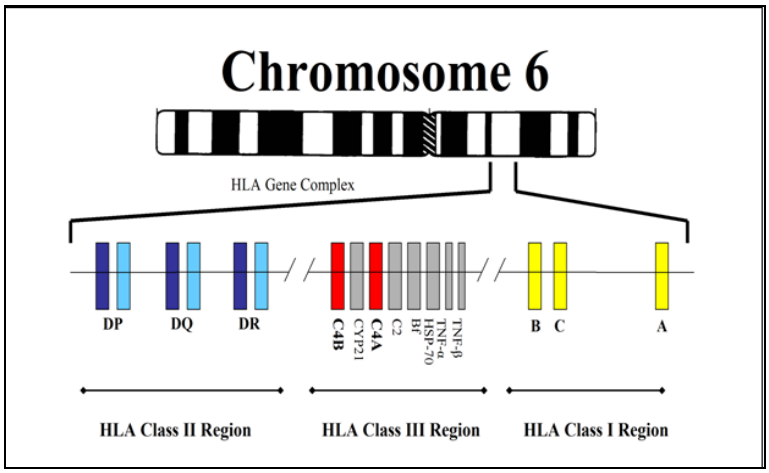

Figure 3: Simplified map of the HLA region on chromosome 6 (Westover *et al.*, 2011).

Table 1: HLA DRB and DQB alleles Genotyped in Asthma Patients

HLA-DRB1*(15)	HLA-DQB1 (11)
*0101-04	*0201-02
*0301-05	*0301/04
*0401-22	*0302
*0701	*03032
*0801-11	*0305
*0901	*0401-02
*1001	*0501-04
*1101-22	*0601
*1201-03	*0602
*1301-22	*0603
*1302	*0604-09
*1401-22	
*1501-05	
*1601-02	
*1701	

Association of DQB1*0503 and the allelic combination DQB1*0201/0301 was found with susceptibility of asthma. Conversely, allele DQB1*0501 and one haplotype DQA1*0101-DQB1*0501-DR1 reported significant protection as found in healthy control subjects in French population (Bignon *et al.,* 1994). HLA DRB1*are found highly associated in an English population study (Moffatt *et al.,* 1999).

2.6 HUMAN LEUKOCYTE ANTIGEN G (HLA-G)

HLA-G is part of the HLA class I paralogues that are heavy chained. This is a heterodimer molecule of class I containing a light chain and a heavy chain (beta-2 microglobulin). It is involved in presenting the foreign antigens to the immune system. In asthmatics, it is upregulated on the bronchial epithelium. It is reported that susceptibility to asthma has been influenced by genetic polymorphism affecting expression of *HLA-G* (Le Page *et al.*, 2013).

2.7 TUMOR NECROSIS FACTOR ALPHA (TNFα)

Tumor necrosis factor (*TNF*) alpha affects airway inflammation and immune response, which are characteristics found in asthma. *TNF* gene maps within cluster on MHC class III region, which clusters on chromosome 6p21. Genetic factors may

influence, and different polymorphisms in the *TNF* gene are associated with TNFα production and TNFα levels may be affected by genetic factors, which result in potential increased risk of asthma. The association of a G-308A variant of TNF-alpha with asthma is found in different populations (Witte *et al.*, 2002)

2.8 CYTOKINES

Different cytokines have also been associated with asthma. The interleukin 4 / interleukin 13 pathway is one of the most intriguing signaling pathways in atopy. In immunological studies, this has been understood that *IL-4* is one of the cytokine mainly involved in immunoglobulin E (IgE) switching in B-cells, animportant feature commonly found in all diseases related to atopy. Presently it has been found that as compared to *IL-4*, *IL-13* is the highly potent mediator of atopic immune responses in the asthma induction. The central cell related to atopy, the T regulatory cell, produces two cytokines *IL-4* and *IL-13*. These two cytokines signal in a same pathway containing sharing between receptor chain (*IL4Ra*) and an intracellular signal transducer (*STAT6*) (Ober and Hoffjan, 2006).

Cytokines are mainly proteins signaling extracellular, usually the size is smaller than 80kD, and mostly are glycosylated. These are produced by various types of cells, which are involved in interactions of one cell with other cell working

through some specific receptors on the target cells surface (Tomita *et al.,* 2004). Following are the cytokines selected for the present study.

2.8.1 Interleukin 10(*IL10*)

Monocytes can produce *IL-10* upon Programmed cell death protein 1 (*PD-1*) which is triggered in these cells (Said *et al.,* 2010). Cytotoxic T-cells release *IL-10* that retards the activity of Natural Killer cells during the response of immune system against any viral infection.

Pro-inflammatory cytokines' synthesis just as *IL-2*, *IFN-γ*, *IL-3*, *TNFα* and *GM-CSF* presented by cells for example macrophages and regulatory T-cells can be inhibited by *IL-10*. The capacity of antigen presenting cells to present antigens is suppressed by *IL-10*; however, it also causes stimulation of certain mast cells and T cells and resultantly stimulates antibody production and B cell maturation.

The effect of functional polymorphisms of *IL10* on allergy and asthma exacerbations is modified by dust mite exposure. Three SNPs in *IL10* (rs1800896, rs3024492, and rs3024496) were found associated with dust mite allergy. The rare allele homozygosity of any of the 3 SNPs was seen associated with more risk of disease (Hunninghake *et al.,* 2008).

2.8.2 Interleukin 1 receptor, type I (*IL1R1*)

It is a key mediator which is involved in most of the cytokine-induced inflammatory and immune system responses. It is the gene, which is localized in a cluster of related cytokine receptor genes on chromosome 2q12. Part of the *IL-33* receptor complex is encoded by *IL1RL1*. Asthma associated genetic variations at the *IL33* and *IL1RL1* loci can be dissected into independent signals with different functional consequences for this pathway, which is basic to pathogenesis of asthma (Grotenboer *et al.*, 2013).

2.8.3 Interleukin 13 (*IL13*)

A class of protein-degrading enzymes is induced by *IL-13*, also called matrix metalloproteinases (MMPs), in lungs airways. These are the enzymes that are needed for induction of egression of effete parenchymal inflammatory cells into the airway lumen from where they are cleared afterwards. Along with several different agents, these are the MMPs, which are induced by *IL-13* as portion of the mechanism that give protection against more inflammation due to allergy, which predisposes to asphyxiation. *IL13* is a 2.9 kb gene characterized by extensive linkage disequilibrium, therefore genotyping some of the polymorphisms gave enough genetic associations assessment. This cytokine is reported to be important for the allergen-induced asthma pathogenesis but operates through some of the

mechanisms that are independent of eosinophils and IgE. Chronic obstructive pulmonary disease (COPD) and asthma are chronic diseases of respiratory system that involve an interaction between environmental and genetic factors. It is suggested that *IL13* has a critical role in pathogenesis of both of these diseases. *IL-13* is seen to be primarily associated with the induction of disease of airways; it also has anti-inflammatory properties. Three of the *IL13* SNPs rs1881457, rs1800925and rs20541 were reported to be associated with the risk of atopy (Bottema *et al.,* 2010). The SNPs rs1295685 and rs20541 were significantly associated with increased IgE levels in pooled analyses of different populations.

2.8.4 Interleukin 33 (*IL33*)

IL-33 receptor was first discovered as a molecule which is like IL-1 receptor (Tominaga, 1989). This was found to be expressed preferentially in Th2 cells and started to attract many researchers working on allergy (Yanagisawa *et al.,* 1997).

2.8.5 Interleukin-18 (*IL18*)

IL-18 is the cytokine which is part of the superfamily of IL-1 and is originated from macrophages and different other cells. By binding to the interleukin-18 receptor the work of *IL-18* starts, and along with IL-12 it promotes cell-mediated immunity following microbial products infection just as lipopolysaccharide (LPS).

IL-18 is well known to initiate severe inflammatory reactions, which leads to the suggestion that it has very important role in many of the inflammatory disorders. *IL-18*, marked as an IFN-γ–producing factor, is a proinflammatory cytokine that has a key role in activation of TH1 cells. *IL-18* may play a critical role to promote immunologic responses and may reflect activity of the disease in mild and moderate asthma attacks (Tanaka *et al.,* 2001).

2.8.6 Interleukin 4 Receptor alpha (*IL4RA*)

IL-4, a pleiotropic cytokine is released by activated Th2 cells and mast cells, and plays a crucial role in immune system responses. The *IL-4* effects are enhanced after binding to high affinity receptor complexes found on hematopoietic as well as non-hematopoietic cells. Hematopoietic responses to *IL-4* are produced by a high affinity receptor complex comprised of the common cytokine γc chain (CD132) and the IL4Rα (CD124) subunit. The IL4Rα subunit in combination with the IL13Rα chain serves as a functional complex for IL-13 in non-hematopoietic cells. *IL4Rα* is a type I transmembrane protein consisting of ser/Pro-rich regions in cytoplasmic domain, same to those located in the GM-CSF receptor ß-chains and the IL-2 receptor. The gene *IL4RA* is localized at chromosome 16p (16p12.1) (Pritchard *et al.,* 1991), a region previously described linked with atopy in several populations (Ober *et al.,* 2000; Deichmann *et al.,* 1998). It is evident that association of *IL4* polymorphisms with total IgE levels and resultantly leading to different phenotypes

of allergy and asthma, along with its ethnical differences have also been seen (Basehore *et al.,* 2004). Specifically, asthma phenotype has been seen to be associated with the promoter region of *IL4* (Rosenwasser and Borish, 1997) and in this region a polymorphism at 33C>T has been reported (Suzuki *et al.,* 1999). *IL-4* works with the help of IL-4 receptor (IL-4R) which has two subunits, an α chain (*IL-4Rα*) and a γ chain (γc) (Nelms *et al.,* 1999; Miloux *et al.,* 1997). *IL-4Rα* is a constituent part of the *IL-4* as well as the IL-13 receptor complexes (Mak and Simard, 1999).

IL-4 and *IL-13*both are the genes, which on their receptors share a common IL-4Rα chain and code cytokines (immunoregulatory proteins) that share their functions. By stimulating IgE synthesis, these cytokines play a central role in allergy (Akdis, 2006). A statistically significant association rs1801275and allergic asthma was reported in a recent meta-analysis of seven studies (Lozaand Chang, 2007).

2.8.7 Thymic Stromal lymphopoietin (*TSLP*)

In humans, the cytokine thymic stromal lymphopoietin (*TSLP*) is found to be related to inflammatory diseases due to allergy. It has been confirmed that *TSLP* is an important initiator of allergic inflammatory diseases for example asthma and atopic dermatitis (Bird, 2005). In many disease states it is reported that *TSLP*

expression is linked to asthma (Ying *et al.,* 2005), atopic dermatitis (Ebner *et al.,* 2007), inflammatory arthritis (Koyama *et al.,* 2007), and eczema and other allergic states (Soumelis and Liu, 2004; Soumelis *et al.,* 2002). A hemopoietic cytokine is encoded by the gene that is supposed to be presented by a receptor complex that is heterodimeric and is constituted of the receptor of thymic stromal lymphopoietin and the IL-7R alpha chain. This primarily affects the cells of myeloid and starts the release of chemokines attracted by T cell from monocytes, and promotes the dendritic cells CD11c (+) maturation step. The product in form of protein enhances response of T helper type 2 (TH2) cells which have association with immune system in different inflammatory diseases, including inflammation due to allergy, asthma, and chronic obstructive pulmonary disease. A *TSLP* gene variant has association with asthma and airway hyperresponsiveness. *TSLP* has an association with allergic rhinitis in asthmatic children. In an analysis two SNPs in *TSLP,* rs2289276 and rs1837253 were significantly associated with a reduced chances of asthma (Hunninghake *et al.,* 2010).

2.9 CHEMOKINES

Chemokines are a group of small cytokines, or signaling proteins secreted by cells. Their name is derived from the ability of inducing direct chemotaxis in nearby responsive cells; they are chemotactic cytokines. Following are the chemokines listed in which polymorphisms are selected for the present study.

2.9.1 Clara cell protein 16 (*CC16)*

Asthma and related phenotypes are found to be linked to chromosome 11q12-13 and this has been indicated in genome-wide searches and candidate gene studies (Cookson *et al.,* 1989; Daniels *et al.,* 1996). Including some other important genes, the gene Clara cell protein 16 (*CC16*) has been identified in this region. It represents various immunomodulatory and anti-inflammatory effects (Singh and Katyal, 1997). The protein CC16 is mainly expressed in the lung and it is likely that its anti-inflammatory effects are to be most pronounced in the airways (Sengler *et al.,* 2003).

2.9.2 Chemokine (C-C motif) ligand 5 (*CCL5*)

CCL5 is chemotactic for eosinophils, T cells and basophils, and plays a critical role in recruiting leukocytes into inflammatory places. This chemokine in humans has been mapped to chromosome 17 (Donlon *et al.,* 1990). By the assistance of specific cytokines (i.e., *IL -2* and *IFN -γ*) that are liberated by T cells, induction of *CCL5* into the activation and proliferation of some of the natural-killer (NK) cells to make CHAK (CC-Chemokine-activated killer) cells (Maghazachi *et al.,* 1996).

2.9.3 C-C motif chemokine 11 (*CCL11*)

This gene is encoded on three exons and is located on chromosome 17 (Kitaura *et al.,* 1996; Hein *et al.,* 1997). The chemoattractant for eosinophils which is the most selective, Eotaxin (*CCL11*), assists peripheral blood eosinophils recruitment into the lung during severe inflammation due to allergy (Rothenberg, 1999; Gonzalo *et al.,* 1998). *CCL11* is localized at a region of chromosome 17q21.1 which is mostly reported to have linkage to asthma in genome-wide linkage studies (Nickel *et al.,* 1997; Koppelman *et al.,* 2002, Barnes *et al.,* 2001). CCL11 locus is a key marker to determine serum total IgE levels between asthmatic patients (Raby *et al.,* 2006).

2.10 BETA 2 ADRENERGIC RECEPTOR (ADRB2)

The *ADRB2* gene present on chromosome 5 is intron less. Various polymorphisms, point mutations, and/or down regulation of this gene are associated with obesity, nocturnal asthma, and diabetes. A study of Scottish asthma patients concluded that rs1042713 (A) alleles are significantly associated with asthma (Basu *et al.,* 2009). Polymorphisms in β-adrenergic receptor gene may also predict the response to asthma therapy. It is very interesting that there is a positive association between the response shown by asthmatic patients to β-adrenergic agonists and the variants of arginine-16in the β-adrenergic receptor gene.

2.11 NITRIC OXIDE SYNTHETASE GENE(NOS)

Nitric oxide (NO) production is regulated by the nitric oxide synthase pathway. Synthesis of NO is from l-arginine by three NO synthase (*NOS*) isoforms. Different genes produce these NOS isoforms. In humans, these have been characterized as: neuronal NOS (nNOS) encoded by the gene *NOS1*, inducible NOS (iNOS) encoded by the gene *NOS2A*, and endothelial NOS (eNOS) encoded by the gene *NOS3*. The intracellular l-arginine availability is a factor that is rate-limiting in NO production (Morris, 2004). These three isoforms of NOS are expressed in airway epithelium (Ricciardolo *et al.*, 2006; Sheffield *et al.*, 2006; Shimokawa and Tsutsui, 2010). Epigenetic differences in NOS genes may alter NO synthesis by affecting NOS function or expression. These changes, in turn, have major influence on the respiratory health outcomes. The endogenous nitric oxide (NO) plays a critical role in regulating physiology of airway functions and is implicated in diseases of airways such as asthma (Ricciardolo *et al.*, 2006; Ghoshand Erzurum, 2011). One of the SNP located in the *NOS3* gene rs1799983 (also known as Glu298Asp, or G894T) was associated with pregnancy-induced hypertension in African women (Hillermann *et al.*, 2005).

2.12 NEUROPEPTIDE S RECEPTOR 1 (NPSR1)

NPSR1 was first specified as a gene susceptible for asthma by positional cloning (Laitinen *et al.,* 2004). The evidence of genetic association was supported by significance in single nucleotide polymorphism (SNP) and associations of haplotypes with asthma in three varying populations. Recently, in seven independent populations , the association of *NPSR1* to asthma and allergy has been replicated (Hersh *et al.,* 2007; Feng *et al.,* 2006; Melen *et al.,* 2005; Kormann *et al.,* 2005; Daley *et al.,* 2009; Castro-Giner *et al.,* 2010;Malerba *et al.,* 2007). It has been reported in different studies that *NPSR1* is involved in inflammatory diseases of skin and intestine (D'Amato *et al.,* 2007; Sundman *et al.,* 2009), traits related to neural system such as sleep and circadian phenotypes (Gottlieb *et al.,* 2007) and anxiety (Leonard *et al.,* 2008).

2.13 TOLL LIKE RECEPTOR (TLR4)

The Toll like receptor present on chromosome 9 plays a basic role in recognition of pathogens and innate immunity activation. The *TLR4* presents some polymorphisms implicated in increased susceptibility to various diseases such as atherosclerosis (Kiechl *et al.,* 2003), asthma (Hold *et al.,* 2007), malaria (Mockenhaupt *et al.,* 2006), and also infection with the *H. pylori* associated with gastric cancer and its precursors (Lorenz *et al.,* 2001). Two SNPs in

TLR4+896A/G (rs4986790) and +1196C/T (rs4986791) have received special attention in some studies, although the results are still controversial (Achyut *et al.*, 2007; Wu *et al.*, 2006;Garza-Gonzalez *et al.*, 2007).

2.14 FC EPSILON RECEPTOR I BETA-CHAIN (FCER1B)

FCER1B gene is located on chromosome 11. An uncertain functional consequence of coding variants of this gene is associated with asthma. A common promoter region polymorphism at the *FCER1B*− 109C/T was identified, although in whole of the coding region no variant was found. This polymorphism in the promoter region is found to be associated with asthma (Hizawa *et al.*, 2000).

2.15 OROSOMUCOID (YEAST)-LIKE 3 (ORMDL3)

It was demonstrated in a genome-wide association study (GWAS) of asthma that several genes on chromosome 17q21 (one of the marker is orosomucoid like 3; *ORMDL3*) were highly and reproducibly associated with asthma in three asthma cohorts of different populations (Moffatt *et al.*, 2007). The confirmation of *ORMDL3* as an asthma-associated gene has been done in additional GWAS studies and in genetic association studies in diverse ethnic backgrounds communities (Bouzigon *et al.*, 2008; Galanter *et al.*, 2008; Moffatt *et al.*, 2010). Presently, the function of *ORMDL3* in the asthma and in lung function is not known. In

independent replication studies this 17q21 locus has shown high association with childhood asthma diagnosis in 3,301 cases from the English population and in 2,320 cases from a cohort of children of German population. These results describe that genetic variants regulating *ORMDL3* expression are determinants of asthma susceptibility in children (Moffatt *et al.*, 2007).

Single-nucleotide polymorphism (SNP)-based genome-wide association study has shown that in Caucasians, genes located at chromosome 17q21 were found to be linked to asthma in childhood but not with atopy, with the highest association being shown for the SNP rs7216389 of the *ORMDL3* gene. In Chinese asthmatics this type of association was not reported. This study showed the frequencies of genotype and allelotype of 10 SNPs at chromosome 17q21, and inquired the relationship between these SNPs and plasma IgE and asthma in southern Chinese children. In this study 315 cases and 192 healthy controls were recruited. In rs7216389, the allele frequency of C allele was varied significantly from 0.232 in controls, 0.389 in Han Chinese to 0.536 in Caucasians. Diagnosis of asthma was associated with rs7216389, rs11650680, whereas rs11650680 was found to be associated with atopy (Leung *et al.*, 2009).

2.16 GASDERMIN A (GSDMA)

GSDMA gene is located at chromosome 17q21.2.The region is thought to be associated with susceptibility to asthma and intermediate phenotypes of asthma,

such as increased IgE levels. In Korean asthmatic children *GSDMA* polymorphisms had an active role in the development of childhood asthma (Yu *et al.,* 2011).

2.17 THROMBOXANE A2 RECEPTOR (TBXA2R)

In a very detailed sequencing of the *TBXA2R* gene in Japanese population rs1131882 (c.795 T>C) has shown association with asthma and various phenotypes related to asthma (Takeuchi *et al.,* 2013). In a study on Korean cerebral infarction patients SNPs in the *TBXA2R*gene rs3786989 (–3372G>C), rs11085026 (+4710T>C) and +4839T>C were analyzed (Sun *et al.,* 2009).

2.18 TRANSFORMING GROWTH FACTOR BETA 1 (TGFB1)

TGF-β1 located on chromosome 19, acts a keypart in monitoring the immunity, and shows various functions on different types of cell, or cells at varied stages of development. Many of the leukocytes (or immune cells) release *TGF-β1* (Letterio and Roberts, 1998). A SNP in the *TGF β 1* gene's promoter region, rs1800469, has been shown association with raised risk for chronic obstructive pulmonary disease (COPD). Although smoking is the major risk factor, smokers with C allele for rs1800469 (or 2 other *TGFB1* SNPs, rs1982073 and rs2241712) were reported to be more likely to develop COPD, based on a study of ~700 Caucasians (Celedón *et al.,* 2004).

2.19 ADISINTEGRIN AND METALLOPROTEINASE DOMAIN-33(ADAM 33)

A member of the ADAM (a disintegrin and metalloprotease domain) family is encoded as ADAM 33. It is a transmembrane protein which is type I and is implicated in bronchial hyperresponsiveness and asthma.*ADAM33* is mapped on chromosome 20p13. It was first discovered by positional cloning in 2002 as susceptibility gene for asthma (Su *et al.,* 2008). Its strong association with asthma was reported in world populations as expression of *ADAM33* is in lung (fibroblasts and bronchial smooth muscle) and lymph nodes. *ADAM33* is an excellent candidate gene for asthma susceptibility. *ADAM33*are highly expressed in smooth muscle cells and airways fibroblast. Any aberration in the action of *ADAM33* leads to functional abnormalities of fibroblasts and smooth muscle cells of airways of lungs. It is more strictly expressed in mesenchymal cells. Association of *ADAM33* with hyper responsiveness in bronchial region and accelerated functional declineof lungs with time strongly recommends it is involved in the structural airway asthma components, for example remodeling (Holgate *et al.,* 2006). Early increased risk of asthma due to SNPs reported at chromosome 17q21 and risk increases due to environmental tobacco smoking (Bouzigon *et al.,* 2008).

2.20 ANGIOTENSIN-CONVERTING ENZYME (ACE)

The gene *ACE* encodes two isozymes. Expression of the somatic isozyme is high in different tissues, primarily in the lung, including vascular endothelial cells, testicular Leydig cells and epithelial kidney cells, whereas the germinal isozyme expression is mainly in sperm. *ACE* gene is an I/D polymorphism in intron 16 of the gene meaning that the presence (I) or absence (D) the carriers of the *ACE* insertion allele of a repeat of alu (Wang *et al.*, 2008). Polymorphisms in *ACE* gene may also predict the response to asthma therapy. An insertion/deletion (I/D) variant of *ACE* is found in different populations. (Eryüksel *et al.*, 2009).

About 153 SNPs reported in *IL4* and some of these are associated with asthma (Noguchi et al., 1998). There are 96 SNPs in *IL13* and 90 in *ADRB2* reported, some have associations with asthma in other world population (Hawkins *et al.*, 2006). Case - control and familial genetic association studies have mainly shown a link between asthma and *ADAM33*. The association of *ADAM33* with hyperresponsiveness in bronchial tract and accelerated lessening in function of lung over time points strongly to the involvement of this gene in the asthma. About 211 SNPs have been reported in *ADAM 33* till date. Few of them have strong association with asthma (Su et al., 2008). In the present study different genes and their SNPs were selected. The purpose is to genotype Pakistani population for major asthma related genes to identify asthma susceptible genes and SNPs.

Table 2: Single Nucleotide Polymorphisms (SNPs) selected for Genotyping in Asthma Patients.

Gene	Chr location	rs #	Allele (minor/major)
IL10	1	rs1800871	T/C
IL10	1	rs1800896	G/A
IL1R1	2	rs10173081	T/C
ADRB2	5	rs1042713	A/G
IL13	5	rs1295685	T/C
IL13	5	rs1800925	T/C
TSLP	5	rs1837253	T/C
IL13	5	rs20541	T/C
TSLP	5	rs2289278	G/C
HLA-G	6	rs1063320	C/G
NOS3	7	rs1799983	T/G
NOS3	7	rs1800779	G/A
NPSR1	7	rs740347	C/G
IL33	9	rs1342326	G/T
TLR4	9	rs4986790	G/A
IL18	11	rs1946518	T/G
FCER1B	11	rs2583476	C/T
CC16	11	rs3741240	A/G
NOS1	12	rs2682826	T/C
IL4RA	16	rs1801275	G/A
IL4RA	16	rs1805011	C/A
ORMDL3	17	rs11650680	T/C
CCL11	17	rs17809012	G/A
CCL5	17	rs1800825	C/T
GSDMA	17	rs3894194	T/C
ORMDL3	17	rs7216389	C/T
ORMDL3	17	rs8079416	T/C
TBXA2R	19	rs1131882	A/G
TFGB1	19	rs1800469	T/C
TBXA2R	19	rs4523	C/T
ADAM33	20	rs2280091	G/A
ADAM33	20	rs528557	G/C
ADAM33	20	rs543749	T/G

Chapter 3

MATERIALS AND METHODS

This study is a case control population based study. Ethical clearance was received by the ethical committee of the parent organization (ERC-08-01) and from the Ethical Committee for the Use of Human Subjects, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi. Written consent from each participant was obtained before taking the samples.

3.1 BLOOD SAMPLE COLLECTION FOR THE PURPOSE OF RESEARCH

Total 854 blood samples including 333 asthma cases and 521 normal healthy controls were recruited for the present study. Cases of asthma were selected for sample collection from the outpatient clinics of Rawalpindi, Islamabad and Lahore. Chest specialists based on clinical examination diagnosed the patients. Normal subjects, as control, were selected from general healthy population. The cases and controls included in the present study have some specific characters due to which they were different from general people. Characteristics of cases and controls are given in Table 3.

3.2 CRITERIA FOR SELECTION OF ASTHMA CASES

Patients and normal healthy controls were selected based on criteria set in the questionnaire filled at the sample collection time having all the required information.

3.2.1 Criteria for Inclusion

i. The Adult patient above age of 12 years and no upper age limit
ii. No gender bias discrimination
iii. Those individuals who show interest and sign the consent form
iv. Patients suffering from asthma

3.2.2 Criteria for Exclusion

i. Individuals less than 10 years of age.
ii. Individuals not willing to sign the informed consent form.
iii. Patients not certainly diagnosed with asthma

Table 3: Characteristic of asthma patients and controls.

	Asthma Patients	**Controls**
Sample size	333	521
Mean Age (Years ± SE)	40 ± 0.93	37 ± 0.77
Males	148 (44.5%)	316 (60.6%)
Females	185 (55.5%)	205 (39.3%)

3.3 DNA EXTRACTION AND QUANTIFICATION

About 4 to 5ml venous blood was drawn in Acid Citrate Dextrose (ACD) vaccutainers by means of sterile syringes. Before genomic DNA extraction blood samples were stored at 4°C. The blood was processed for genomic DNA isolation by the method of phenol chloroform extraction (Sambrook and Russel, 2001). For the extraction of DNA 15 mL cell lysis buffer (155 mM Ammonium Chloride, 0.1 mM EDTA, 10mM Potassium Bicarbonate) was added in a 50mL tube along with blood sample. These samples were placed on ice for 30 minutes and were centrifuged at 1200 rpm for 10 minutes for complete lysis of RBCs in the samples. The supernatant was discarded and above procedure was repeated with fresh cell lysis buffer. The WBCs pellet obtained was resuspended in 4.75 mL of STE buffer (100mM Sodium chloride, 50mM Tris, 1mM EDTA). To this mixture 250µL 10% SDS was added dropwise to lyse the WBCs and 10µL of proteinase K (20 mg / mL) was added to denature the proteins. The tubes were then incubated in water bath at 55 °C over night.

After digesting overnight with proteinase K, in each tube equal quantity of Equilibrated Phenol (pH=8) Chloroform and Isoamylalcohol (24:1) mixture (1:1) was added. After shaking well for 10 minutes so that a homogenous mixture is formed, tubes were placed on ice for ten minutes. These tubes were then centrifuged for thirty minutes at 3200 rpm. In the extracted aqueous layer 5mL ice

cold isopropanol along with 600µL 10M ammonium acetate was added and shaken well until the DNA was visible in the form of white threads.

The tubes were placed at -20 °C over night. These tubes were centrifuged at 3200 rpm for 50 minutes. DNA was settled down in the form of pellet. The DNA pellet was washed with 5mL of ice cold 70 per cent ethanol. The pellet was resuspended in 500-800 µL of 10 mM TrisHCl depending on pellet size. The stocks of DNA were stored at -20 °C. All the mentioned centrifugations were done at 4°C. DNA stocks were quantified by absorbance measurement at 260nm in Ultravtec spectrophotometer. The 1:50 dilution of DNA (6 µL DNA in 294 µL deionized H_2O) was prepared in a glass tube and measured the absorbance at 260nm and 280nm respectively.

Calculation of stock DNA concentration was done by using the following standard formula for double stranded DNA:

Absorbance measured at 260nm × Dilution Factor (50) × Correction Factor (50) = DNA Concentration µg/ mL

3.4 SNP SELECTION

All genes reported for asthma were retrieved at NCBI. From the list the genes strongly associated with asthma in other populations were selected. From these

genes the SNPs already reported to be highly associated with asthma were searched using SNP flanking options present at NCBI's (http://www.ncbi.nlm.nih.gov/) BLAST search engine and sequences were retrieved.

3.5 GENOTYPING

For genotyping, 34 SNPs in 23 different genes were selected. Selection favored SNPs with evidence for association with asthma risk reported in other world populations and these were not studied in Pakistani population.

ACE I/D polymorphism was also studied in cases and controls. This rs4646994 is an insertion/deletion of an Alu repetitive element in an intron of the ACE gene. Alleles having the insertion are denoted as "I" alleles and "D" alleles are the ones lacking this repetitive element. Additionally, HLA class II was also typed in Pakistani asthmatic cases and controls. In these DRB and DQB loci were typed in the cases and controls.

Three different methods were used for SNP genotyping: TaqMan genotyping to obtain data on individual genotypes, iPLEX genotyping to analyze several SNPs simultaneously and one SNP with allele specific PCR. For 28 SNPs 2 iPLEXs were designed; one for 20 SNPs and the other for 8 SNPs. In iPLEX designed for 20SNPs, 2 SNPs did not work and for those we designed TaqMan assay and rest of

the six SNPs were also typed by TaqMan assay. This genotyping was done in Department of Human Genetics, University of Chicago, Chicago, USA.

3.6 iPLEX

The iPLEX genotyping by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) on the MassArray platform (Sequenom) was used in this present study (Gabriel *et al.*, 2009). The SNPs selected for iPLEX are listed in table 4.

This technique has the following steps.

3.6.1 Multiplex PCR amplification

MassArray Assay design software (ver. 3.1) was used to design the primers. PCR reactions were done in 384-well plates; reaction contained, in a volume of 5µL, 60nM of the corresponding primers, 20ng genomicDNA, 1.625mM of $MgCl_2$, 500µM of dNTPs and 0.5U Hot Star Taq (Qiagen). The conditions for PCR were: 94°C for15 min, following 45 cycles of 94 °C (20 sec), 56 °C (30 sec), 72 °C (60 sec), and finally the extension at 72 °C for 3 min. The PCR primers list for amplification reaction is given in Appendix I and II.

After amplification to see amplification, some of the samples were separated on 2 per cent agarose gel before doing extension reaction (Figure 4).

After extension reaction, it was run on Sequenom software TYPER analyzer to visualize all assays in the multiplex reaction. In that the plate was visualized as colored traffic lights (Figure5). These signals were shown by different SNPs and the mass to intensity peaks were visualized and these told about the presence or absence of base at that particular place (Figure 6).

3.6.2 Digestion by Shrimp alkaline phosphatase (SAP reaction)

Excess of deoxyribonucleotide triphosphates were dephoshorylated by adding 0.3 USAP (iPLEX Gold Reaction Kit; Sequenom) to the PCR reaction following the incubation for 40 min at 37°C. Denaturation of shrimp alkaline phosphatase was done at 85°C for 3 min.

Table 4: Single nucleotide polymorphisms done by iPLEX Technique.

Gene	Chr location	rs #	Allele (minor/major)
IL10	1	rs1800871	T/C
IL10	1	rs1800896	G/A
ADRB2	5	rs1042713	A/G
IL13	5	rs1295685	T/C
IL13	5	rs1800925	T/C
IL13	5	rs20541	T/C
NOS3	7	rs1799983	T/G
NOS3	7	rs1800779	G/A
NPSR1	7	rs740347	C/G
IL33	9	rs1342326	G/T
IL18	11	rs1946518	T/G
FCER1B	11	rs2583476	C/T
NOS1	12	rs2682826	T/C
IL4RA	16	rs1801275	G/A
IL4RA	16	rs1805011	C/A
ORMDL3	17	rs11650680	T/C
CCL11	17	rs17809012	G/A
CCL5	17	rs1800825	C/T
GSDMA	17	rs3894194	T/C
ORMDL3	17	rs8079416	T/C
TBXA2R	19	rs1131882	A/G
TFGB1	19	rs1800469	T/C
TBXA2R	19	rs4523	C/T
ADAM33	20	rs2280091	G/A
ADAM33	20	rs528557	G/C
ADAM33	20	rs543749	T/G

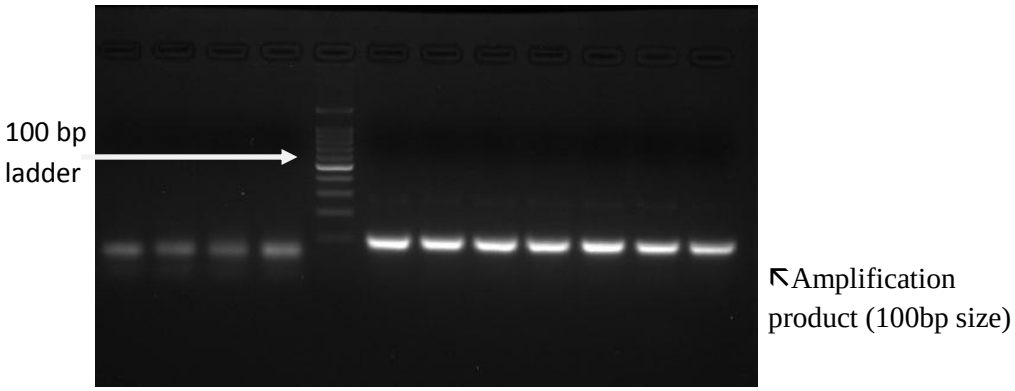

Figure 4: Amplification product shown on 2% agarose gel. Gel shows just the amplification and here just amplification product presence is enough.

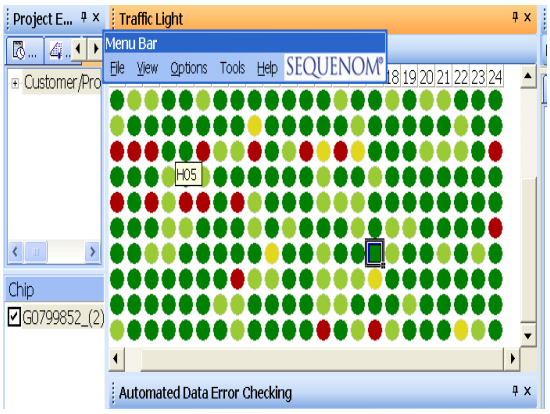

Figure 5: The traffic lights of signals of different assays in the multiplex reaction.

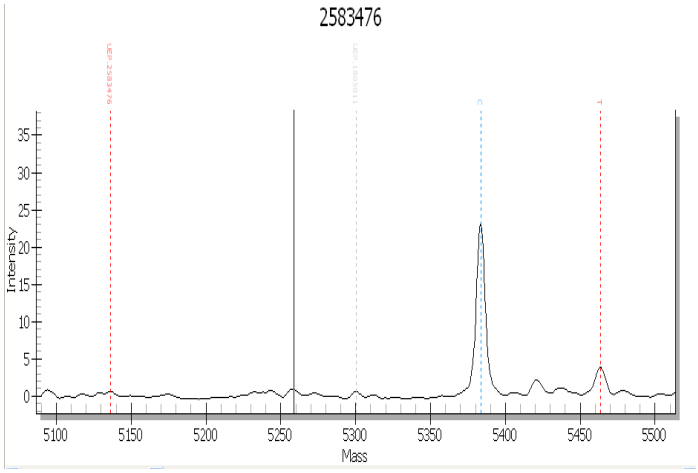

Figure 6: **In rs2583476 the peak shown at 5383.5 Daltons for C call at that place.**

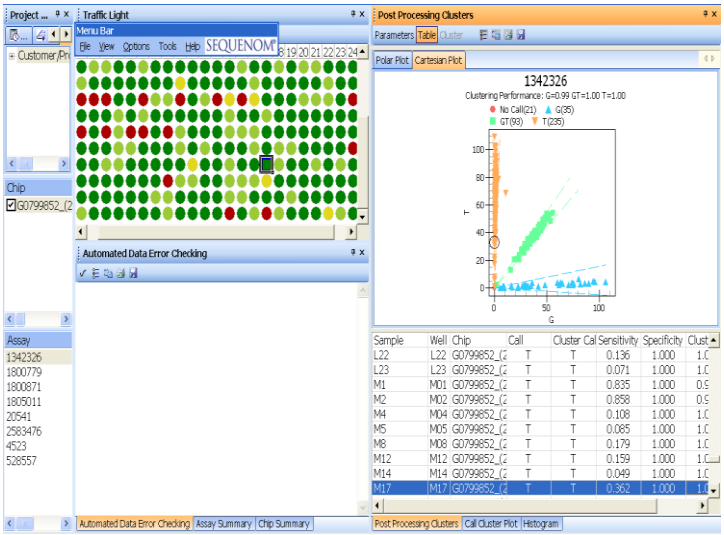

Figure 7: **The results shown after analysis by Typer analyzer showing rs1342326, the homozygous and heterozygous and no calls along with signals shown in excel file.**

3.6.3 Extension of Primers for iPLEX

MassArray design software was used to design primers for extension reaction. The reaction mix for iPLEX consisted of 0.2X iPLEX buffer, 0.2 µL iPLEX termination mix, 0.041 µL iPLEX enzyme (iPLEX Gold Reaction Kit; Sequenom), and 0.056 µL to 0.113 µL primer. The primers for extension reaction were divided into a high-mass group and a low mass group and the high-mass group concentration was doubled. For iPLEX 2-step 200 short-cycle programs were used for PCR amplification. Denaturation of the sample was done at 94 °C for 30 sec., annealing at 52 °C for 5 sec. and extension at 80 °C for 5 sec. Then there were 4 more repetitions of annealing and extension cycles making a total of 5 cycles, looping back to a denaturation step at 94 °C for 5 sec., and finally entered again to the 5-cycled loop of annealing and extension reaction. These five annealing and extension steps with the single denaturing step were repeated additionally 39 times making a total of 40. The final extension for 3 min. was done at 72 °C. The list of extension primers is given in Appendix III and IV.

3.6.4 Resin Cleaning

Desaltation of iPLEX reaction products were done by adding 6 mg resin (Clean Resin; Sequenom) and 25 µL water to each well. After incubation at room temperature for 30 min, the reaction mixture was centrifuged at 3500 g for 5min.

3.6.5 Data analysis and MALDI-TOF MS analysis

Using a Nanodispenser the samples were dispensed onto a 384 SpectroCHIPArray, and in MassArray Compact mass spectrometer SpectroCHIPArrays were introduced. Spectra acquisition automation was performed using Spectroacquire (Sequenom). The analysis of data was performed by using the MassArray Typer software ver. 3.4.

3.7 TAQMAN

TaqMan allelic discrimination assays were performed on the ABI7900HT Fast Real Time sequence detection system (Applied Biosystems). The method was first reported in 1991 (Holland *et al.*, 1991). The allelic discrimination assay contains two different fluorescently labelled probes, one for each allele of the SNP. Each probe consists of an oligonucleotide with a fluorescent reporter dye at the 5' end and a quencher at the 3' end. During PCR amplification, the TaqMan probes hybridize only to perfectly matching DNA, and Taq polymerase with 5' to 3' exonuclease activity cleaves the hybridized probe. This cleavage separates the quencher from the reporter, allowing the fluorescence of the reporter dye to be detected. The measured emission represents the genotype of each sample. The PCR conditions used for amplification were 95°C for 10 min, and 40cycles of 92°C

for 15s and 60°C for 1 min. The six SNPs done by TaqMan assay are listed in Table 5.

3.8 HLA TYPING FOR HLA CLASS II ALLELES (DRB1*AND DQB1*)

HLA class II alleles were screened in asthma cases and controls by means of sequence specific primers (PCR-SSP). Primer mixes for HLA-DRB and DQB1 were made according to the HLA photo typing method by Bunce *et al.* (1995). The primer mixes, 10 xPCR buffer, and TDMH reagents were made in bulk and kept at -20°C for storage purpose. All reagents were prepared in autoclaved deionized water. The 10 x PCR buffer contained 670 mM Tris base pH 8.8, 66 mM ammonium sulphate and one percent tween 20. The TDMH consists of 2.6xPCR buffer, 460 μM of dNTPS and 6.25 mM $MgCl_2$. TDMH was freshly prepared according to requirement and stored in 5mL falcon tubes at 4 °C.

Table 5: Single nucleotide polymorphisms genotyped by Taqman Technique.

Gene	**Chr location**	**rs #**	**Literature cited**	**Allele (minor/major)**
IL1R1	2	rs10173081	16	T/C
TSLP	5	rs1837253	24,25	T/C
TSLP	5	rs2289278	27,28	G/C
HLA-G	6	rs1063320	9	C/G
TLR4	9	rs4986790	33	G/A
CC16	11	rs3741240	30,31	A/G
ORMDL3	17	rs7216389	11	C/T

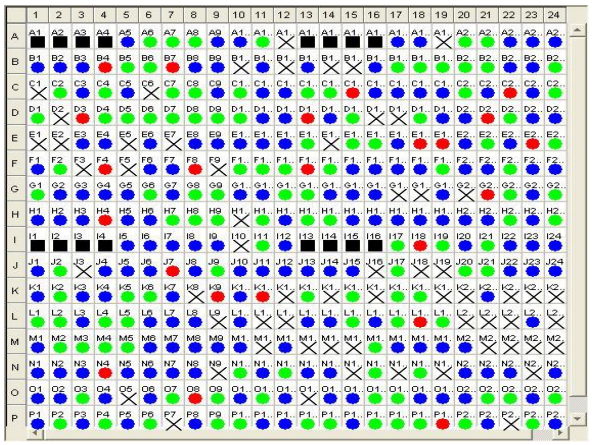

Figure 8: The signals of different genotypes in the TaqMan reaction showing homozygous, heterozygous and not amplified samples.

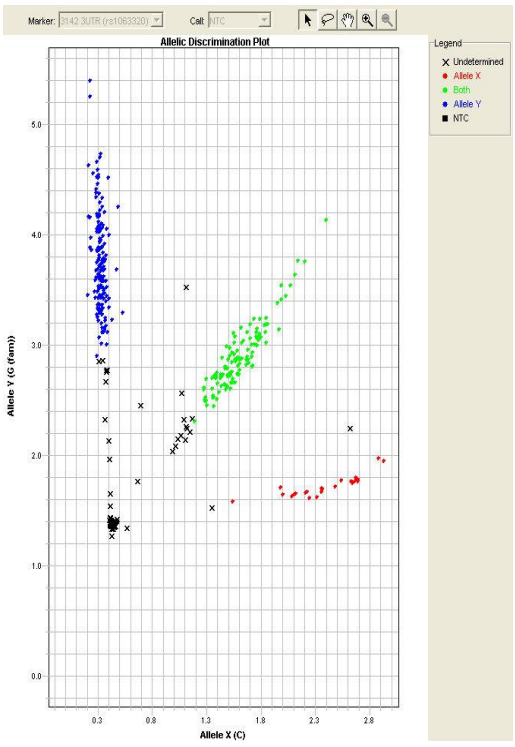

Figure 9: The signals of different genotypes in the TaqMan reaction showing homozygotes and .heterozygotes and not amplified samples.

3.8.1 Polymerase Chain Reaction with Sequence Specific Primers for HLA Class II Typing

Polymerase chain reaction with sequence specific primers (PCR-SSP) was done for each patient and control sample, using the method as given by Bunce *et al.* (1995). PCR was done in 13 μL final volume in 96 well plates. Class II primer mixes (5 μL) were aliquoted in to labeled plates using the multichannel dispenser. Master mixes was prepared, that included 5 μL of TDMH, 0.187 units of Taq polymerase, 0.1 ng of DNA sample per reaction, and then to each primer mix in the 96 well plate, 8 μl of the master mix was added. The plates were properly sealed with the sealing films. PCR was done, using cycling conditions of one min at 96 °C following 5 cycles of 25 sec at 96°C, 45 sec at 70 °C and 72 °C for 45 sec, then 21 cycles of 96 °C for 25 sec, 65 °C for 50 sec and 72 °C for 45 sec, following the 4 cycles of 25 sec at 96 °C, 55 °C for one min and 72 °C for two min and last step was 1 cycle for 10 min at 72 °C.

3.8.2 Agarose Gel Electrophoresis

The amplimers were separated on 2 per cent agarose gel, prepared in 0.5 X Tris-Acetate-EDTA, (TAE) buffer. 5 μL of ethidium bromide (5 μg / mL) was added into the gels to visualize the DNA bands under UV lights. In each sample

before running on the gel, 5 μL of loading dye orange G (6 X) was added. The gels were electrophoresed for about one hour at 120 volts until the dye migrated at least 3 cm. DNA ladder of 100bp was used as standard marker to identify alleles according to size. The gels were photographed under UV light. Positive band for different alleles were scored and the haplotypes were determined (Figure 10).

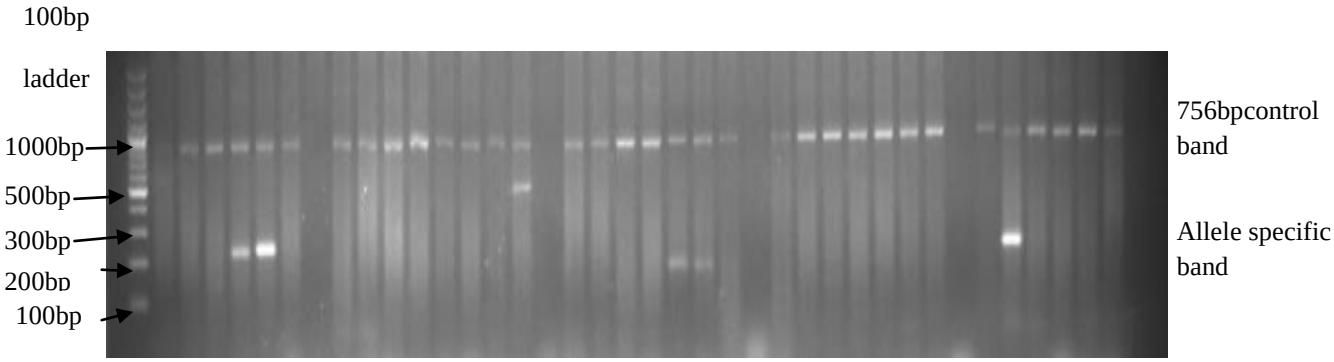

Figure 10: Agarose Gel Electrophoresis showing results of HLA typing class II.

3.9 ANGIOTENSIN- CONVERTING-ENZYME INSERTION/DELETION POLYMORPHISM

Angiotensin-converting enzyme (ACE) Alu I/D polymorphism in intron 16 were studied using primers sequences as given in Table 6. A 15 µL volume was used for each PCR reaction, each reaction contained 1 µM of both primer, 1x of 10xPCR buffer, 0.45 µM of $MgCl_2$, 200 µM of dNTPS and 1 U of *Taq* DNA polymerase (Fermentas EU) and 30 ng of DNA as final concentration per reaction. PCR cycling parameters were, 1 cycle 94 °C for three min, following 35 cycles of 94 °C for 45 sec, 58 °C for one min, and 72 °C for 45 sec and of 1 cycle of 72 °C for 10 min (Batzer *et al.*, 1996).

Amplicons were separated on two percent agarose gels and after staining with ethidum bromide agarose, gels were visualized under UV transilluminator. The fragment D allele is at the size of 190 bp while the I allele is seen at the size of 490 bp. Both size bands were present in ID heterozygotes. DNA ladder of 100 bp was used as standard (Figure 11).

3.10 TUMOR NECROSIS FACTOR ALPHA 308 POLYMORPHISM

Tumor Necrosis Factor (TNF) alpha 308 was typed using PCR-SSP by using primers sequences as given in Table 7. The sequence specific primers of TNF alpha 308 consisting of one forward and two reverse primers were used. In this β-Globin was used as positive controls. A 15 µL volume was used for each PCR reaction, each reaction contained 1 µM of both primer, 1x of 10xPCR buffer, 0.45 µM of $MgCl_2$, 200 µM of dNTPS and 1 U of *Taq* polymerase (Fermentas EU) and 30 ng of DNA as final concentration per reaction. PCR cycling parameters were, one cycle at 95 °C for 5 min, following 31 cycles at 95 °C for 90 sec, 61 °C for 150 s, at 72 °C for one min and of one cycle for 10 min at 72 °C.

Table 6: Primer sequences of ACE I/D polymorphism.

Forward	5' – CTGGAGACCACTCCCATCCTTTCT-3'
Reverse	5'-GATGTGGCCATCACATTCGTCAGAT-3'

Table 7: Primer sequences of TNF alpha 308 polymorphism.

TNF-144/-164	5'-TCTCGGTTTCTTCTCCATCG-3'
TNF-A1-328/-308G	5'-ATAGGTTTTGAGGGGCATGG-3'
TNF-A2-320/-308A	5'-ATAGGTTTTGAGGGGCATGA-3'
BETA-GLOBIN(A)	5'-ACACAACTGTGTTCACTAGC-3'
BETA-GLOBIN(B)	5'-CAACTTCATCCACGTTCACC-3'

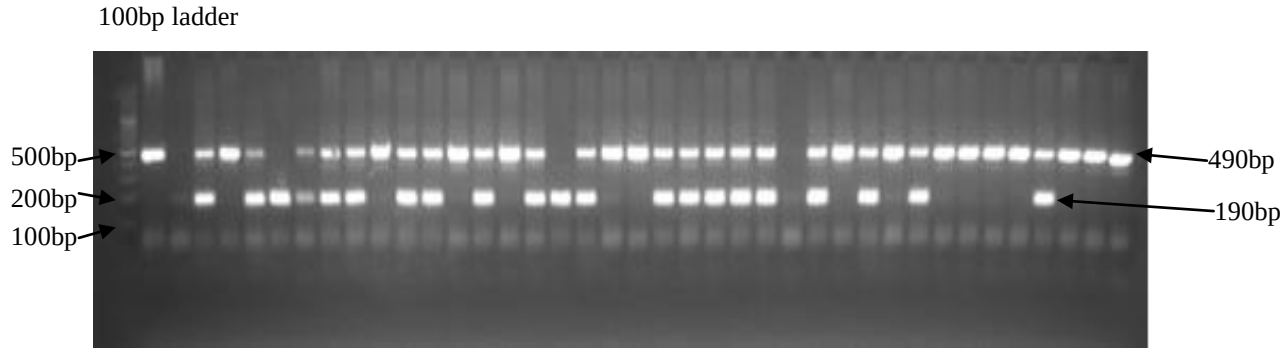

Figure 11: Agarose Gel Electrophoresis showing results of ACE I/D PCR products.

The amplimers were separated by using two percent agarose gel electrophoresis and after staining with ethidum bromide agarose gels were visualized under UV transilluminator. The fragment of size 185 bp represented TNF alpha 308 G/A polymorphism while a fragment of 110 bp represented the positive control of beta Globin. DNA ladder of 100 bp was used as standard in the gel (Figure 12).

3.11 STATISTICAL ANALYSIS

Phenotype characteristics are described as the means ± standard error (SE).

Association testing

Association of individual genetic variants and asthma was calculated using logistic regression, adjusted for age, and sex, assuming an additive genetic model. The results are presented in the form of odds ratio (OR) along with 95 per cent confidence intervals (CI).

The analysis of variance was calculated by Statistical Package for Social Sciences (SPSS) for significant variation between class II allelic frequencies and haplotypes two locus in the asthma cases and controls (Voelki and Gerber, 1999).

For a particular locus where only one allele was found that sample was said to be homozygous. Calculations for odd ratio (OR) and 95 per cent confidence interval (CI) were done by means of calculator for Odds Ratios (http:// www.hutchon.net/ConfidOR.htm; Bland and Altman, 2000). The OR significance was calculated by using the 2 x 2 chi-square contingency- test with Yates' correction for continuity (http://faculty.vassar.edu/lowry/ VassarStats.html). Hardy-Weinberg equilibrium (HWE) was calculated by Arlequin Version 3.0 software (http://cmpg.unibe.ch/software/ arlequin3; Schneider *et al.*, 2000)

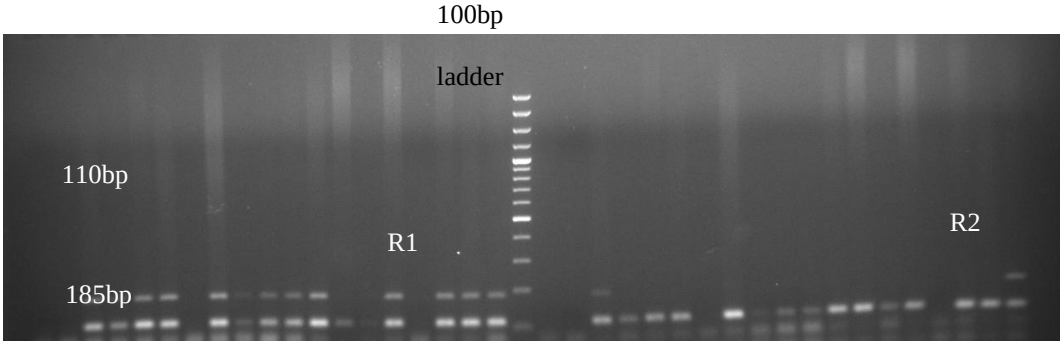

Figure 12: Agarose Gel Electrophoresis showing results of *TNF α* 308 PCR products.

Comparison of the case and control groups was done by Fisher Exact test. In case of ACE I/D polymorphism and TNF alpha 308 polymorphism the OR with 95% CI were calculated by means of the online statistical software package for 2-way Contingency table analysis (http://vassarstats.net/odds2x2.html).

3.12 META-ANALYSIS

Electronic databases like Med line and PubMed were searched up to February 2014 for all genetic studies involving HLA DRB1*0701 and HLA DQB1*06 typing in asthma in humans in different studies all around the world. Studies selected in our meta-analysis were all done on human beings, they were case control based studies, data for detailed genotypes frequency of patients and controls were available. Meta-analysis was done using Comprehensive Meta-analysis version 2 Biostat, Englewood, NJ, 2004. The association of HLA DRB1*0701 and HLA DQB1*06 with asthma was estimated for each study by OR and 95 per cent CI.

Chapter 4

RESULTS AND DISCUSSION

In present study, 854 blood samples were collected from asthma patients and healthy individuals. Samples were genotyped for different associated genes SNPs in cases and controls. The case-control specific gender distributions are provided in (Table 3). Mean age of our population was 40 $\pm$ 0.93 in cases and 37 $\pm$ 0.77 in controls.

4.1 GENOTYPE/ALLELEFREQUENCIES AND HARDY-WEINBERG EQUILIBRIUM IN CASES AND CONTROLS

In the present study, total 34 different SNPs of different genes were analyzed by using three different techniques; iPLEX, Taqman, and sequence specific PCR. The results of these SNPs are shown in Table 8. The allele and genotype frequencies of all the samples including cases and controls were analyzed for Hardy-Weinberg equilibrium (HWE). The genotype distributions for all SNPs were consistent with **HWE** except for rs740347 (*NPSR1*) and rs1946518 (*IL18*) having values HW_P= 0.000701 and 0.007414 respectively (Table 8). These two SNPs were therefore excluded from the study and remaining 32 SNPs were included for further analysis.

The allele and genotype frequencies of all SNPs were also separately calculated for the cases and controls as shown in tables 9 and 10. The HWE values for SNPs rs1946518 (*IL18*), rs740347 (*NPSR1*) and rs2280091 (*ADAM33*) showing deviation from HWE (p=0.02) were excluded from study controls. In asthma cases, two SNPs, rs1342326 (*IL33*) and rs740347 (*NPSR1*) deviated from HWE (p=0.04) and thus were not included in the final analyses.

Table 8: SNPs with their call rates, minor allele frequency and Hardy-Weinberg Equilibrium p-values.

S. No	Gene	SNP	Call Rate	Minor Allele Frequency (MAF)	HWE p-value
1	*IL1R1*	rs10173081	0.99	0.06	0.99
2	*TLR4*	rs4986790	1.00	0.11	0.22
3	*IL10*	rs1800871	0.97	0.40	0.23
4	*IL10*	rs1800896	0.97	0.26	0.22
5	*IL13*	rs1295685	0.97	0.31	0.15
6	*IL13*	rs1800925	0.96	0.22	0.38
7	*IL13*	rs20541	0.96	0.30	0.05
8	*IL18*	rs1946518*	0.95	0.34	0.01
9	*IL33*	rs1342326	0.97	0.17	0.09
10	*IL4RA*	rs1801275	0.97	0.20	0.25
11	*IL4RA*	rs1805011	0.97	0.05	0.05
12	*TSLP*	rs1837253	0.99	0.32	0.56
13	*TSLP*	rs2289278	0.92	0.09	0.35
14	*TGF-β1*	rs1800469	0.96	0.35	0.05
15	*CC16*	rs3741240	0.98	0.40	0.62
16	*CCL5*	rs1800825	0.97	0.03	0.43
17	*CCL11*	rs17809012	0.96	0.45	0.62
18	*ADRB2*	rs1042713	0.96	0.43	0.57
19	*NPSR1*	rs740347*	0.96	0.08	0.00
20	*FCER1B*	rs2583476	0.97	0.48	0.19
21	*GSDMA*	rs3894194	0.96	0.49	0.77
22	*NOS1*	rs2682826	0.97	0.29	0.33
23	*NOS3*	rs1799983	0.97	0.19	0.65
24	*NOS3*	rs1800779	0.96	0.20	0.23
25	*HLA-G*	rs1063320	0.96	0.26	0.09
26	*ORMDL3*	rs11650680	0.97	0.22	0.60
27	*ORMDL3*	rs8079416	0.96	0.49	0.78
28	*ORMDL3*	rs7216389	0.90	0.41	0.20
29	*TBXA2R*	rs4523	0.96	0.48	0.38
30	*TBXA2R*	rs1131882	0.96	0.19	0.25
31	*ADAM33*	rs2280091	0.94	0.19	0.06
32	*ADAM33*	rs528557	0.91	0.39	0.16
33	*ADAM33*	rs543749	0.94	0.19	0.33

*The SNPs that are not in Hardy-Weinberg equilibrium.

4.2 ASSOCIATION OF GENOTYPES AND ALLELES WITH ASTHMA

4.2.1 Association of SNPs in Interleukin Genes

In interleukins different genes were selected. One SNP each of *IL1R1*, *TLR4*, and *IL33* gene, two SNPs in *IL10* and *IL4RA* and three SNPs of *IL13* were genotyped in cases and controls For *IL1R1*gene, one SNP rs10173081 was studied in a case control model. As mentioned in table 11, it was found that no allele was associated with asthma significantly (p=0.14). Similarly for*TLR4,* one SNP rs4986790 was typed in 337 patients and 169 controls and no significant association with asthma was observed (p=0.63). The *IL-1* family has been involved in inflammatory and immunologic responses (Dinarello, 2009). Members contain activators and suppressors of inflammation. Interleukin-1 receptors (IL-1Rs) and Toll-like receptors (TLRs) are members of a large superfamily of phylogenetically conserved proteins involved in innate immunity and inflammation (Mantovani *et al.,* 2007). The common characteristics of these two receptor families include their presence in the cytoplasmic region of a conserved sequence called Toll/IL-1R (TIR) domain (Dinarello, 2009; O'Neill, 2008). The *IL-1R*/*TLR*-driven immune response also has an essential role in the induction and/or regulation of allergic inflammation and disease exacerbations.

For *IL10,* two SNPs, rs1800871 and rs1800896were typed. For rs1800871, 324 patients and 176 controls and for rs1800896, 326 patients and 172 controls were included. The rs1800871 has no significant association with asthma in Pakistani population (p=0.11). In rs1800896 minor allele G had significant

association with risk of asthma in our studied Pakistani population (p=0.04). The G allele carriers are at increased odds of disease as compared to non-carriers (OR=1.38, 95% CI=1.01-1.88)).

IL-10 is known to be important in immuno-regulation and is considered as an immune-suppressive factor (Lyon *et al.,* 2004; Guzowski *et al.,* 2005; Rees *et al.,* 2002). Low levels of *IL-10* expression have a role on the pathogenesis of asthma (Makela *et al.,* 2000; Tarzi *et al.,* 2006). On the other hand, high level of *IL-10* from regulatory T cells has a protective effect against airway hyper-reactivity and inflammation (Stampfli *et al.,* 1999). The polymorphism rs1800896 conferred susceptibility to asthma in East Asians and adult asthmatics. It lies within a putative ETS-like transcription factor binding site, it has been reported that G position may be associated with a higher expression of the *IL-10* gene (Mormann *et al.,* 2004; Gruber *et al.,* 2008). The association with the G allele in the Pakistani population is consistent with results of studies in Indian (Chatterjee *et al.,* 2005), Egyptian (Hussein *et al.,* 2011) and studies from other regions (Chung, 2001; Asadullah *et al.,* 2003; Barnes, 2002). A few studies have also reported the opposite allele as in Korean population the association is with A allele (Park *et al.,* 2004).

For *IL13,* three SNPs rs1295685, rs1800925 and rs20541 were analyzed. Among these only rs1800925 SNPs minor T allele has shown significant association towards disease (p=0.03) increasing disease risk (OR=1.45 with 95% CI=1.04-2.02) in allele carriers. For the other two SNPs rs1295685 (p=0.68) and

rs20541 (p=0.81) there was no association in our study population. Interleukin (*IL-13*) is a critical mediator in the pathogenesis of allergic inflammation (Hershey, 2003). This cytokine up-regulates major histocompatibility complex class II expression and promotes IgE isotype switching. This cytokine is found to be critical to the pathogenesis of allergen-induced asthma but operates through mechanisms independent of IgE (Leonardo *et al.*, 2008). It has also been reported that the rs1800925 (-1112C/T) polymorphism resulted in enhanced promoter activity (Vladich *et al.,* 2005). In spite of the importance of *IL-13* in asthma (Karp *et al.*,1998; Noakes *et al.*, 2003; Feleszko *et al.,* 2006) some studies failed to show an association between *IL13* polymorphisms and asthma phenotypes (Leung *et al.*, 2001; Hakonarson and Wjst,2001), possibly because of different prevalence of environmental risk factors such as tobacco smoke exposure. Beghé (Beghé *et al.*, 2010) reported associations of rs1800925, rs1295685 and rs20541 in *IL13* with both atopy and asthma. These are different SNPs which are located in *IL13* and all are not in LD with each other. In the present study these three SNPs were genotyped in Pakistani cases and controls however only rs1800925 showed significant disease association. In all above mentioned studies, including ours, the T allele is associated with disease susceptibility.

In *IL33,* SNP rs1342326 was typed in 325 patients and 175 controls and no significant association of this SNP was observed with asthma (p=0.64). *IL-33* induces the expression of *IL-4*, *IL-5*, and *IL-13* and leads to severe pathologic changes in mucosal organs (Schmitz *et al.,* 2005). Mice injected with human *IL-33* exhibit impressive pathologic changes in the arterial walls, lungs, and intestinal

tissues (Schmitz *et al.*, 2005). Of particular relevance to the concept of *IL-33*-driven Th2 response is the prominent eosinophilic infiltration in lung tissue. Airway smooth muscle cells have *IL-33* expression in both the protein and mRNA levels. *IL-33* expression increases in bronchial biopsies in asthmatic subjects compared to controls, as well as subjects with severe asthma (Préfontaine *et al.*, 2009).

In *IL4RA* two SNPs, rs1801275 (328 patients and 172 controls) and rs1805011 (322 patients and 176 controls) were typed. Both SNPs lacked significance in association towards disease in our studied Pakistani samples with p values=0.64 and 0.21 respectively.

4.2.2 Association of SNPs in Other Cytokines

Two SNPs, rs1837253 and rs2289278 of TSLP gene were typed in 327/307 patients and 185/167 controls respectively. Both SNPs lacked association (p=0.23 and 0.92 respectively) with asthma risk in our population. For TFGB1 gene, rs1800469 SNP was genotyped in 324 patient and 170 controls. This SNP also lacked association towards disease (p=0.54).

Transforming growth factor-β1 (*TGF-β1*) is a multifunctional cytokine that may influence asthma by modulating allergic airway inflammation and airway remodeling (Duvernelle *et al.*, 2003). It is important in growth, transformation, tissue repair, fibrosis and the modulation of immune inflammatory responses

(Blobe *et al.,* 2000). Its levels in bronchoalveolar lavage (BAL) fluid are higher in asthma patients and increase further in response to allergen exposure compared with healthy control subjects (Redington *et al.,* 1997). Several SNPs in this cytokine have been studied for association with asthma and atopy, including rs1800469. This SNP in the *TGF-β1*promoter appears to influence *TGF-β1* blood levels (Grainger *et al.,* 1999; Suthanthiran *et al.,* 2000) and gene expression in the lung (Silverman *et al.,* 2004). Association of this and other *TGF-β1* SNPs with wheezing illness in infants (Hoffjan *et al.,* 2004), asthma diagnosis (Mak *et al.,* 2006; Silverman *et al.,* 2004), severity (Pulleyn *et al.,* 2001) and increased IgE in asthmatic children (Hobbs *et al.,* 1998) have been found in some studies (Buckova *et al.,* 2001; Heinzmann *et al.,* 2005). In the present study no association between rs1800469 in *TGF-β1*and asthma samples was reported. The T allele at this SNP has been associated with asthma or related phenotypes in many previous studies (Celedón *et al.,* 2004; Nagpal *et al.,* 2005; Li *et al.,* 2007).

4.2.3 Association of SNPs in Chemokines

For CC16 gene, one SNP rs3741240 was typed in 326 patients and 179 controls. This marker lacked significant association with asthma in our study population (p=0.61). Similarly, rs1800825 SNP of *CCL5* and rs17809012 SNP of *CCL11* were genotyped for their association with asthma. None of the SNPs showed significant association with disease having p values 0.53 and 0.39 respectively.

4.2.4 Association of ADRB2, FCER1B, GSDMA Genes SNPs with Asthma

Three SNPs, rs1042713, rs2583476, rs3894194 from ADRB2, FCER1B and GSDMA, genes each were also genotyped in present study. None of the SNPs showed significant association towards asthma with p values 1.0, 0.09 and 0.65 respectively.

4.2.5 Association of SNPs in NOS1, NOS3, HLA-G Genes

Three SNPs, rs2682826 NOS1 and rs1799983 and rs1800779 from NOS3 genes were also genotyped in our asthma cases and healthy controls. No significant association (p=0.60, 0.36 and 0.67 respectively) of asthma with these SNPs was observed in our study population. Similar results were obtained for rs1063320 SNP of HLA-G gene (p=0.39).

4.2.6 Association of SNPs in Genes ORMDL3, TBXA2R

For ORMDL3 gene, three SNPs rs11650680, rs8079416 and rs7216389 were typed. In all three SNPs no significant result was obtained having p values=0.54, 0.46 and 0.47 respectively (Table 11). For TBXA2R gene, two SNPs rs1131882 and rs4523 were genotyped in 325 patients and 171 controls. The minor allele of rs1131882 (*TBXA2R*) showed significant association (p=0.05) with disease (Table 11). However, an OR of 0.73 (95% CI=0.52-1.01) seems to indicate some protective role of this SNP in our population. In order to fully explore exact role

of this SNPs as disease marker (risk/protective), large scale studies need to be conducted. The second *TBXA2R* SNP rs4523, we did not find any significant association with disease (p=0.71).

The thromboxane A2 receptor (*TBXA2R*) is a potent broncho- and vasoconstrictor and is associated with leukotriene synthesis. It is involved in prostaglandin and leukotriene pathways, and has diverse physiological and pathophysiological actions related to allergies, modulation of acquired immunity, atherogenesis, and neovascularization (Nakahata, 2008). Polymorphisms in the TBXA2R gene have been associated with urticarial (Palikhe *et al.*, 2011). This study investigated associations between asthma- and *TBXA2R* polymorphism. We have found a borderline association between rs1131882 (*TBXA2R)* and asthma in Pakistani population. Its significance is at borderline. It shows here that A allele is associated with protection in Pakistani population. In Japanese population genetic variants in the TBXA2R gene were also associated with asthma-related phenotypes (Takeuchi *et al.,* 2013). Thromboxane pathways may therefore play important roles in airway inflammation and remodeling in asthma patients.

4.2.7 Association of SNPs in ADAM33 Gene

In ADAM33 gene, three SNPs rs2280091, rs528557 and rs543749 were studied for disease association. For rs528557, 300 patients and 167 controls were typed while for rs543749, 318 patients and 167 controls were typed. In both these

SNPs no significant associations were observed having p=1.0 and 0.31 respectively.

The rs2280091 (*ADAM33*) has significant p value of 0.03 with reference to minor allelic condition as shown in table 11. Its significance is also shown in the odds ratio value and 95 per cent confidence interval (OR = 0.69; 95% CI = 0.50-0.97). In this study 318 patients and 169 controls were genotyped. The first strong genetic evidence suggests that genes in non-allergic, non-immune pathways may play important roles in asthma pathogenesis was the report of *ADAM33* as the first positionally cloned asthma gene (Eerdewegh *et al.,* 2002; Ober and Yao, 2011). *ADAM33* encodes a disintegrin and metalloprotein-33 protein that participates in the bronchial remodeling process in asthma (Vergaraa *et al.,* 2010). Asthma is a complex disease, but learning more about how SNP mutations in *ADAM33* give rise to asthmatic conditions will provide important clues in treating asthma. The ADAM33 gene has been found to be expressed in lung fibroblasts, and bronchial smooth muscle, but not in bronchial epithelial cells. This indicates a strong link between the *ADAM33* gene and asthmatic conditions. Strong association of rs2280091 *(ADAM33)* in Taiwanese (Chiang *et al.,* 2012) and Saudi population was reported (Al-Khayyat *et al.,* 2012). It was also reported to be associated with Asian population (Liang *et al.,* 2013). In the present study we have also found a very strong association of this polymorphism showing association of G with protection which is in contrast to above mentioned study. This difference may be due to different genetic makeup of our population.

From the SNPs based studies it was concluded that variations in TBXA2R, ADAM33, IL10 and IL13 genes seem to offer disease protection in the Pakistani population. Further studies will be needed to replicate these associations in the Pakistani population and to elucidate the mechanism for these observations.

4.3 GENDER BASED ASSOCIATION BETWEEN SNPs AND ASTHMA

All age and gender adjusted genotyped data was also explored for gender based disease association.

4.3.1 SNP Genotypes Distribution in Male Subjects

For all genotyped SNPs no gender specific disease association was seen except FCER1B gene. For FCER1B gene SNP, rs2583476 has shown significant genotype distribution in males. As shown in table12, the asthmatic male gender had higher TT genotype counts as compared to controls. The homozygous TT and CC genotype frequencies in cases were; TT 35 per cent and GG 24.3 per cent as compared with controls; CC 24 per cent and GG 27.7per cent. These results clearly indicate that in our sample population of male gender TT genotype is highly prevalent in asthma patients as compared to healthy controls, whereas, GG genotype is not much different between males in cases and controls. Gender specific association with this SNP in asthma patients has not been reported in different world populations.

4.3.2 SNP Genotypes Distribution in Female Subjects

In female gender, the only significant association found was in ORMDL3 gene SNP rs11650680. As shown in table 12, heterozygous CT genotype is more prevalent in female asthma cases as compared to controls female population. In case control data, the CT genotype frequency in female cases is 36 per cent and 23 per cent in control female subjects. These results clearly show that CT genotype of ORMDL3 gene SNP rs11650680 is highly prevalent in female asthma cases as compared to healthy controls in our study population.

4.3.3 Gender Based Comparison of SNP Genotypes among Asthma Cases and controls

A comparison was also made between male and female subjects with regards to genotype frequencies of studied SNPs under case-control model (Table 12). For FCER1B gene SNP rs2583476 significant difference existed in genotype frequencies among male and female cases (OR=1.86, 95% CI=1.09-3.17, p=0.01). The odds ratio results clearly indicate that male subjects are at higher risk of asthma as compared to female counter-parts. Genotype frequencies also depicted high prevalence of risk allele in males as compared to females. In case of ORMDL3 gene SNP rs11650680, our results indicate that female asthma subjects carriers of CT genotype are at higher risk of disease as compared to male asthmatic subjects (OR=1.99, 95% CI=1.02-3.89, p=0.03).

4.4 ASSOCIATION OF SNPs WITH AGE IN ASTHMA

Concerning age, our study population was distributed among three age groups: < 18 years, 18-40 years and >40 years. Genotype frequencies in each group are listed in Table 13. Of all 31 SNPs genotyped in our population, only one SNP rs1800896 (*IL10*) showed significant association with age (p=0.0067). Based on rs1800896 (*IL10*) genotype analysis, 18-40 years age group is at the highest risk of disease (OR=10.69, 95% CI=4.74-24.12) as compared to >40 years of age. There is a considerable difference in the cases and controls with as shown in table 13.

Table 9: Allele and genotype frequencies in controls and the Hardy-Weinberg p-values.

S.No.	rs #	Gene	Allele (min/maj)	Genotypes			Genotype Frequencies (%)			Allele Frequencies (%)		HWE (p-value)
1	rs1800871	*IL10*	T/C	C/C	C/T	T/T	0.33	0.48	0.19	0.57	0.43	0.78
2	rs1800896	*IL10*	G/A	A/A	A/G	G/G	0.58	0.36	0.05	0.76	0.24	1.00
3	rs1295685	*IL13*	T/C	C/C	C/T	T/T	0.49	0.4	0.11	0.69	0.31	0.42
4	rs1800925	*IL13*	T/C	C/C	C/T	T/T	0.66	0.31	0.03	0.81	0.19	0.82
5	rs20541	*IL13*	T/C	C/C	C/T	T/T	0.5	0.4	0.11	0.69	0.31	0.41
6	rs1946518*	*IL18*	T/G	G/G	G/T	T/T	0.5	0.36	0.14	0.68	0.32	0.02
7	rs1342326	*IL33*	G/T	G/G	T/G	T/T	0.03	0.28	0.69	0.83	0.17	1.00
8	rs1801275	*IL4RA*	G/A	A/A	A/G	G/G	0.66	0.29	0.05	0.8	0.2	0.37
9	rs1805011	*IL4RA*	C/A	A/A	A/C	C/C	0.94	0.05	0	0.97	0.03	0.18

continued

Table 9 Page 2

S.No.	rs #	Gene	Allele (min/maj)	Genotypes			Genotype Frequencies (%)			Allele Frequencies (%)		HWE (p-value)
10	rs1800469	*TFGB1*	T/C	C/C	C/T	T/T	0.42	0.42	0.16	0.63	0.37	0.13
11	rs1800825	*CCL5*	C/T	C/C	T/C	T/T	0	0.05	0.95	0.98	0.02	1.00
12	rs17809012	*CCL11*	G/A	A/A	A/G	G/G	0.32	0.47	0.21	0.56	0.44	0.57
13	rs1042713	*ADRB2*	A/G	A/A	G/A	G/G	0.2	0.46	0.34	0.57	0.43	0.39
14	rs740347	*NPSR1**	C/G	C/C	G/C	G/G	0.02	0.11	0.87	0.92	0.08	0.02
15	rs2583476	*FCER1B*	C/T	C/C	T/C	T/T	0.27	0.46	0.27	0.5	0.5	0.21
16	rs3894194	*GSDMA*	T/C	C/C	C/T	T/T	0.27	0.48	0.25	0.51	0.49	0.48
17	rs2682826	*NOS1*	T/C	C/C	C/T	T/T	0.52	0.4	0.07	0.73	0.27	1.00
18	rs1799983	*NOS3*	T/G	G/G	G/T	T/T	0.64	0.32	0.04	0.8	0.2	1.00
19	rs1800779	*NOS3*	G/A	A/A	A/G	G/G	0.67	0.29	0.04	0.81	0.19	0.65

Table 9 Page 3

S.No.	rs #	Gene	Allele (min/maj)	Genotypes			Genotype Frequencies (%)			Allele Frequencies (%)		HWE (p-value)
20	rs11650680	*ORMDL3*	T/C	C/C	C/T	T/T	0.63	0.32	0.05	0.79	0.21	0.68
21	rs8079416	*ORMDL3*	T/C	C/C	C/T	T/T	0.27	0.47	0.26	0.5	0.5	0.48
22	rs4523	*TBXA2R*	C/T	C/C	T/C	T/T	0.26	0.46	0.29	0.51	0.49	0.21
23	rs1131882	*TBXA2R*	A/G	A/A	G/A	G/G	0.07	0.29	0.65	0.79	0.21	0.06
24	rs2280091*	*ADAM33*	G/A	A/A	A/G	G/G	0.62	0.29	0.08	0.77	0.23	0.02
25	rs528557	*ADAM33*	G/C	C/C	C/G	G/G	0.36	0.48	0.16	0.6	0.4	1.00
26	rs543749	*ADAM33*	T/G	G/G	G/T	T/T	0.7	0.26	0.04	0.83	0.17	0.21

*The SNPs that are not in Hardy-Weinberg equilibrium.

Table 10: Allele and genotype frequencies in cases and the Hardy-Weinberg p-values.

S.No.	SNP ID	Gene	Allele (min/maj)	Genotypes			Genotype Frequencies %			Allele Frequencies %		HWE (p-value)
1	rs1800871	*IL10*	T/C	C/C	C/T	T/T	0.39	0.44	0.17	0.61	0.39	0.20
2	rs1800896	*IL10*	G/A	A/A	A/G	G/G	0.54	0.37	0.09	0.72	0.28	0.17
3	rs1295685	*IL13*	T/C	C/C	C/T	T/T	0.49	0.41	0.11	0.69	0.31	0.37
4	rs1800925	*IL13*	T/C	C/C	C/T	T/T	0.61	0.33	0.06	0.77	0.23	0.21
5	rs20541	*IL13*	T/C	C/C	C/T	T/T	0.52	0.37	0.11	0.71	0.29	0.11
6	rs1946518	*IL18*	T/G	G/G	G/T	T/T	0.45	0.41	0.14	0.65	0.35	0.14
7	rs1342326*	*IL33*	G/T	G/G	T/G	T/T	0.04	0.24	0.72	0.84	0.16	0.04
8	rs1801275	*IL4RA*	G/A	A/A	A/G	G/G	0.64	0.32	0.05	0.8	0.2	0.61
9	rs1805011	*IL4RA*	C/A	A/A	A/C	C/C	0.89	0.1	0.01	0.94	0.06	0.09
10	rs1800469	*TFGB1*	T/C	C/C	C/T	T/T	0.45	0.41	0.14	0.65	0.35	0.14

continued

Table 10 Page 2

S.No.	SNP ID	Gene	Allele (min/maj)	Genotypes			Genotype Frequencies %			Allele Frequencies %		HWE (p-value)
11	rs1800825	*CCL5*	C/T	C/C	T/C	T/T	0	0.06	0.94	0.97	0.03	0.31
12	rs17809012	*CCL11*	G/A	A/A	A/G	G/G	0.23	0.51	0.27	0.48	0.52	0.82
13	rs1042713	*ADRB2*	A/G	A/A	G/A	G/G	0.17	0.53	0.3	0.56	0.44	0.18
14	rs740347*	*NPSR1*	C/G	C/C	G/C	G/G	0.02	0.13	0.85	0.92	0.08	0.04
15	rs2583476	*FCER1B*	C/T	C/C	T/C	T/T	0.22	0.48	0.29	0.54	0.46	0.58
16	rs3894194	*GSDMA*	T/C	C/C	C/T	T/T	0.26	0.5	0.24	0.51	0.49	0.91
17	rs2682826	*NOS1*	T/C	C/C	C/T	T/T	0.47	0.45	0.08	0.7	0.3	0.24
18	rs1799983	*NOS3*	T/G	G/G	G/T	T/T	0.68	0.28	0.04	0.82	0.18	0.57
19	rs1800779	*NOS3*	G/A	A/A	A/G	G/G	0.64	0.3	0.05	0.8	0.2	0.24
20	rs11650680	*ORMDL3*	T/C	C/C	C/T	T/T	0.6	0.35	0.05	0.78	0.22	0.87
21	rs8079416	*ORMDL3*	T/C	C/C	C/T	T/T	0.27	0.51	0.23	0.52	0.48	0.82

Table 10 Page 3

S.No.	SNP ID	Gene	Allele (min/maj)	Genotypes			Genotype Frequencies %			Allele Frequencies %		HWE (p-value)
22	rs4523	*TBXA2R*	C/T	C/C	T/C	T/T	0.23	0.5	0.28	0.52	0.48	1.00
23	rs1131882	*TBXA2R*	A/G	A/A	G/A	G/G	0.02	0.29	0.69	0.83	0.17	0.55
24	rs2280091	*ADAM33*	G/A	A/A	A/G	G/G	0.69	0.28	0.03	0.83	0.17	1.00
25	rs528557	*ADAM33*	G/C	C/C	C/G	G/G	0.4	0.42	0.18	0.61	0.39	0.07
26	rs543749	*ADAM33*	T/G	G/G	G/T	T/T	0.64	0.32	0.04	0.8	0.2	0.73

Table 11: Allele and genotype frequencies in cases and controls and Odds Ratio with p values.

(Gene) SNP	Sample	Minor Allele	Major Allele	Homozygous Minor	Heterozygous	Homozygous Major	Odds Ratio	95%CI	*p*-value
IL1R1		T	C	TT	CT	CC			
rs10173081	case	36	624	1	34	295	0.68	(0.41-1.14)	0.14
	control	28	332	1	26	153			
TLR4		G	A	GG	AG	AA			
rs4986790	case	70	604	7	56	274	0.91	(0.60-1.36)	0.63
	control	42	328	1	40	144			
IL10		T	C	TT	CT	CC			
rs1800871	case	252	396	55	142	127	0.81	(0.62-1.05)	0.11
	control	155	197	33	89	54			

continued

Table 11 Page 2

(Gene) SNP	Sample	Minor Allele	Major Allele	Homozygous Minor	Heterozygous	Homozygous Major	Odds Ratio	95%CI	*p*-value
		G	A	GG	AG	AA			
rs1800896	case	179	473	30	119	177	**1.38**	**(1.01-1.88)**	**0.04**
	control	74	270	7	60	105			
IL13		T	C	TT	CT	CC			
rs1295685	case	206	450	36	134	158	1.06	(0.80-1.41)	0.68
	control	103	239	16	71	84			
		T	C	TT	CT	CC			
rs1800925	case	149	497	21	107	195	**1.45**	**(1.04-2.02)**	**0.03**
	control	59	285	3	53	116			
		T	C	TT	CT	CC			
rs20541	case	191	449	35	121	164	0.97	(0.73-1.28)	0.81
	control	107	243	17	73	85			

Table 11 Page 3

(Gene) SNP	Sample	Minor Allele	Major Allele	Homozygous Minor	Heterozygous	Homozygous Major	Odds Ratio	95%CI	*p*-value
IL18		T	G	TT	GT	GG			
rs1946518	case	223	421	44	135	143	1.19	(0.90-1.58)	0.22
	control	104	234	23	58	88			
IL33		G	T	GG	GT	TT			
rs1342326	case	104	546	14	76	235	0.92	(0.65-1.30)	0.64
	control	60	290	5	50	120			
IL4RA		G	A	GG	AG	AA			
rs1801275	case	134	522	15	104	209	1.08	(0.78-1.50)	0.64
	control	66	278	10	46	116			
		C	A	CC	AC	AA			
rs1805011	case	39	605	3	33	286	1.68	(0.88-3.19)	0.11
	control	13	339	1	11	164			

Table 11 Page 4

(Gene) SNP	Sample	Minor Allele	Major Allele	Homozygous Minor	Heterozygous	Homozygous Major	Odds Ratio	95%CI	*p*-value
TSLP		T	C	TT	CT	CC			
rs1837253	case	199	455	28	143	156	0.85	(0.65-1.11)	0.23
	control	126	244	20	86	79			
TSLP		G	C	GG	CG	CC			
rs2289278	case	54	560	1	52	254	0.98	(0.61-1.56)	0.92
	control	30	304	4	22	141			
TGF-β1		T	C	TT	CT	CC			
rs1800469	case	220	428	44	132	148	0.92	(0.70-1.21)	0.54
	control	122	218	26	70	74			

Table 11 Page 5

(Gene) SNP	Sample	Minor Allele	Major Allele	Homozygous Minor	Heterozygous	Homozygous Major	Odds Ratio	95%CI	*p*-value
CC16		A	G	AA	AG	GG			
rs3741240	case	259	393	53	153	120	0.94	(0.72-1.22)	0.61
	control	148	210	31	86	62			
CCL5		C	T	CC	CT	TT			
rs1800825	case	22	636	1	20	308	1.29	(0.59-2.83)	0.53
	control	9	335	0	9	163			
CCL11		G	A	GG	AG	AA			
rs17809012	case	298	344	69	160	92	1.12	(0.86-1.46)	0.39
	control	149	193	34	81	56			

Table 11 Page 6

(Gene) SNP	Sample	Minor Allele	Major Allele	Homozygous Minor	Heterozygous	Homozygous Major	Odds Ratio	95%CI	*p*-value
ADRB2		A	G	AA	AG	GG			
rs1042713	case	284	364	56	172	96	1.00	(0.77-1.30)	1.00
	control	150	192	37	76	58			
NPSR1		C	G	CC	CG	GG			
rs740347	case	53	595	5	43	276	1.13	(0.69-1.85)	0.63
	control	25	317	3	19	149			
FCER1B		C	T	CC	CT	TT			
rs2583476	case	297	349	70	157	96	0.80	(0.62-1.04)	0.09
	control	180	170	48	84	43			

Table 11 Page 7

(Gene) SNP	Sample	Minor Allele	Major Allele	Homozygous Minor	Heterozygous	Homozygous Major	Odds Ratio	95%CI	*p*-value
GSDMA		T	C	TT	CT	CC			
rs3894194	case	317	329	77	163	83	0.94	(0.72-1.22)	0.65
	control	171	167	48	75	46			
NOS1		T	C	TT	CT	CC			
rs2682826	case	196	456	25	146	155	1.08	(0.81-1.44)	0.60
	control	98	246	12	74	86			
NOS3		T	G	TT	GT	GG			
rs1799983	case	114	540	12	90	225	0.86	(0.61-1.20)	0.36
	control	68	276	7	54	111			
		G	A	GG	AG	AA			
rs1800779	case	132	510	17	98	206	1.07	(0.77-1.49)	0.67
	control	68	282	7	54	114			

Table 11 Page 8

(Gene) SNP	Sample	Minor Allele	Major Allele	Homozygous Minor	Heterozygous	Homozygous Major	Odds Ratio	95%CI	*p*-value
HLA-G		C	G	CC	CG				
rs1063320	case	161	501	25	111	195	0.88	(0.66-1.18)	0.39
	control	98	268	16	66	101			
ORMDL3		T	C	TT	CT	CC			
rs11650680	case	146	508	17	112	198	1.11	(0.80-1.52)	0.54
	control	71	273	8	55	109			
		T	C	TT	CT	CC			
rs8079416	case	310	338	73	164	87	0.91	(0.70-1.18)	0.46
	control	171	169	48	75	47			
		C	T	CC	CT	TT			
rs7216389	case	244	368	51	142	113	0.91	(0.69-1.19)	0.47
	control	137	187	33	71	58			

Table 11 Page 9

(Gene) SNP	Sample	Minor Allele	Major Allele	Homozygous Minor	Heterozygous	Homozygous Major	Odds Ratio	95%CI	*p*-value
TBXA2R		C	T	CC	CT	TT			
rs4523	case	301	335	71	159	88	0.95	(0.73-1.24)	0.71
	control	170	180	46	78	51			
TBXA2R		A	G	AA	AG	GG			
rs1131882	case	110	540	7	96	222	0.73	(0.52-1.01)	0.05
	control	75	267	13	49	109			
ADAM33		G	A	GG	AG	AA			
rs2280091	case	105	531	9	87	222	**0.69**	**(0.50-0.97)**	**0.03**
	control	75	263	15	45	109			
		G	C	GG	CG	CC			
rs528557	case	232	368	53	126	121	1.00	(0.76-1.32)	1.00
	control	129	205	25	79	63			

Table 11 Page 10

(Gene) SNP	Sample	Minor Allele	Major Allele	Homozygous Minor	Heterozygous	Homozygous Major	Odds Ratio	95%CI	*p*-value
ADAM33		T	G	TT	GT	GG			
rs543749	case	128	508	13	102	203	1.17	(0.83-1.65)	0.31
	control	59	275	8	43	116			

Table 12: Genotype distribution among male and female gender in cases and controls and Odds Ratio with p values.

SNP	Gene	Genotypes	Female			Male			p-value
			cases	controls	OR(95%CI)	cases	controls	OR(95%CI)	
rs1800871	*IL10*	C/C	64	41	1	64	29	1.41 (0.78-2.55)	0.24
		C/T	87	53	1.05 (0.63-1.77)	58	47	0.79 (0.46-1.37)	
		T/T	32	25	0.82 (0.43-1.58)	23	14	1.05 (0.49-2.28)	
rs1800896	*IL10*	A/A	98	64	1	77	55	0.91 (0.57-1.46)	0.43
		A/G	68	45	0.99 (0.60-1.61)	52	29	1.17 (0.67-2.04)	
		G/G	13	7	1.21 (0.46-3.20)	17	4	2.78 (0.89-8.63)	
rs1295685	*IL13*	C/C	82	63	1	78	36	1.66 (1.00-2.78)	0.58
		C/T	78	40	1.50 (0.91-2.48)	55	42	1.01 (0.60-1.69)	
		T/T	20	12	1.28 (0.58-2.81)	15	10	1.15 (0.49-2.74)	

continued

Table 12 Page 2

SNP	Gene	Genotypes	Female			Male			p-value
			cases	controls	OR(95%CI)	cases	controls	OR(95%CI)	
rs1800925	*IL13*	C/C	111	77	1	86	57	1.05 (0.67-1.63)	0.044
		C/T	55	33	1.16 (0.69-1.95)	52	31	1.16 (0.68-1.98)	
		T/T	11	6	1.27 (0.45-3.58)	10	0	---	
rs1801275	*IL4RA*	A/A	111	74	1	98	60	1.09 (0.70-1.68)	
		A/G	61	38	1.07(0.65-1.77)	43	22	1.30 (0.72-2.36)	0.71
		G/G	8	4	1.33(0.39-4.59)	7	6	0.78 (0.25-2.41)	
rs1946518	*IL18*	G/G	81	62	1	63	38	1.27 (0.75-2.14)	
		G/T	74	34	1.67 (0.99-2.81)	60	39	1.18 (0.70-1.98)	0.17
		T/T	21	17	0.95 (0.46-1.94)	24	11	1.67 (0.76-3.67)	

Table 12 Page 3

SNP	Gene	Genotypes	Female			Male			p-value
			cases	controls	OR(95%CI)	cases	controls	OR(95%CI)	
rs2280091	*ADAM33*	A/A	121	72	1	98	53	1.10 (0.71-1.71)	
		A/G	46	31	0.88 (0.51-1.52)	44	28	0.94 (0.54-1.63)	0.99
		G/G	6	12	0.30 (0.11-0.83)	3	5	0.36 (0.08-1.54)	
rs2682826	*NOS1*	C/C	80	62	1	73	45	1.26 (0.76-2.07)	
		C/T	85	43	1.53 (0.93-2.51)	62	39	1.23 (0.73-2.07)	0.29
		T/T	14	11	0.99 (0.42-2.32)	11	4	2.13 (0.65-7.02)	
rs20541	*IL13*	C/C	90	64	1	78	39	1.42 (0.86-2.35)	0.24
		C/T	70	42	1.19 (0.72-1.95)	51	41	0.88 (0.53-1.49)	
		T/T	20	13	1.09 (0.51-2.36)	14	9	1.11 (0.45-2.71)	

Table 12 Page 4

SNP	Gene	Genotypes	Female			Male			p-value
			cases	controls	OR(95%CI)	cases	controls	OR(95%CI)	
rs3894194	*GSDMA*	C/C	44	30	1	39	25	1.06 (0.54-2.11)	
		C/T	90	51	1.20 (0.68-2.14)	73	45	1.11 (0.61-2.00)	0.49
		T/T	43	34	0.86 (0.45-1.65)	34	17	1.36 (0.65-2.87)	
rs8079416	*ORMDL3*	C/C	48	29	1	39	25	0.94 (0.48-1.86)	
		C/T	90	51	1.07 (0.60-1.89)	74	45	0.99 (0.55-1.79)	0.28
		T/T	39	36	0.65 (0.34-1.25)	34	17	1.21 (0.58-2.54)	
rs11650680	*ORMDL3*	C/C	106	82	1	91	46	1.53 (0.97-2.42)	
		C/T	65	27	**1.86 (1.09-3.17)**	48	39	0.95 (0.57-1.59)	**0.01**
		T/T	8	7	0.88 (0.31-2.54)	9	3	2.32 (0.61-8.85)	

Table 12 Page 5

SNP	Gene	Genotypes	Female			Male			p-value
			cases	controls	OR(95%CI)	cases	controls	OR(95%CI)	
rs17809012	*CCL11*	A/A	39	36	1	34	30	1.05 (0.54-2.04)	
		A/G	90	56	1.48 (0.85-2.60)	74	40	1.71 (0.94-3.09)	0.97
		G/G	48	24	1.85 (0.95-3.60)	39	18	2.00 (0.97-4.11)	
rs4523	*TBXA2R*	T/T	53	31	1	36	28	0.75 (0.39-1.46)	
		C/T	90	54	0.97 (0.56-1.70)	70	40	1.02 (0.57-1.85)	0.34
		C/C	36	32	0.66 (0.34-1.26)	37	21	1.03 (0.51-2.07)	
rs528557	*ADAM33*	C/C	73	41	1	48	31	0.87 (0.48-1.57)	
		C/G	66	54	0.69 (0.41-1.16)	63	42	0.84 (0.49-1.46)	0.66
		G/G	32	20	0.90 (0.46-1.77)	22	11	1.12 (0.50-2.55)	

Table 12 Page 6

SNP	Gene	Genotypes	Female			Male			p-value
			cases	controls	OR(95%CI)	cases	controls	OR(95%CI)	
rs1342326	*IL33*	T/T	140	85	1	96	58	1.00 (0.66-1.53)	
		G/T	35	29	0.73 (0.42-1.28)	43	30	0.87 (0.51-1.49)	0.92
		G/G	9	4	1.37 (0.41-4.57)	5	2	1.52 (0.29-8.00)	
rs1800779	*NOS3*	A/A	123	81	1	86	58	0.98 (0.63-1.51)	
		A/G	51	32	1.05 (0.62-1.77)	48	29	1.09 (0.64-1.87)	0.61
		G/G	7	5	0.92 (0.28-3.00)	10	3	2.20 (0.59-8.22)	
rs1805011	*IL4RA*	A/A	161	116	1	128	81	1.14 (0.79-1.64)	
		A/C	20	3	4.80 (1.39-16.54)	13	8	1.17 (0.47-2.92)	0.086
		C/C	1	0	---	2	1	1.44 (0.13-16.08)	

Table 12 Page 7

SNP	Gene	Genotypes	Female			Male			p-value
			cases	controls	OR(95%CI)	cases	controls	OR(95%CI)	
rs2583476	*FCER1B*	T/T	42	34	1	54	22	**1.99 (1.02-3.89)**	
		C/T	102	52	1.59 (0.91-2.79)	55	43	1.04 (0.57-1.89)	**0.033**
		C/C	38	32	0.96 (0.50-1.85)	35	25	1.13 (0.57-2.25)	
rs543749	*ADAM33*	G/G	117	77	1	88	63	0.92 (0.60-1.42)	
		G/T	48	31	1.02 (0.60-1.74)	54	20	1.78 (0.99-3.20)	0.29
		T/T	9	5	1.18 (0.38-3.67)	5	3	1.10 (0.25-4.72)	
rs740347	*NPSR1/GPRA*	G/G	147	103	1	129	73	1.24 (0.85-1.81)	
		C/G	26	11	1.66 (0.78-3.50)	16	12	0.93 (0.42-2.06)	0.19
		C/C	3	1	2.10 (0.22-20.49)	2	3	0.47 (0.08-2.85)	

Table 12 Page 8

SNP	Gene	Genotypes	Female			Male			p-value
			cases	controls	OR(95%CI)	cases	controls	OR(95%CI)	
rs1042713	*ADRB2*	G/G	65	40	1	32	30	0.66 (0.35-1.24)	
		A/G	90	56	0.99 (0.59-1.66)	83	37	1.38 (0.79-2.40)	0.18
		A/A	25	20	0.77 (0.38-1.56)	31	20	0.95 (0.48-1.89)	
rs1131882	*TBXA2R*	G/G	122	76	1	102	55	1.16 (0.75-1.79)	
		A/G	51	34	0.93 (0.56-1.57)	44	24	1.14 (0.64-2.03)	0.37
		A/A	5	6	0.52 (0.15-1.76)	2	8	0.16 (0.03-0.75)	
rs1799983	*NOS3*	G/G	123	78	1	99	53	1.18 (0.76-1.84)	
		G/T	49	34	0.91 (0.54-1.54)	43	31	0.88 (0.51-1.51)	0.78
		T/T	7	4	1.11 (0.31-3.92)	5	4	0.79 (0.21-3.04)	

Table 12 Page 9

SNP	Gene	Genotypes	Female			Male			p-value
			cases	controls	OR(95%CI)	cases	controls	OR(95%CI)	
rs1800469	*TFGB1*	C/C	81	42	1	64	43	0.77 (0.45-1.32)	
		C/T	74	54	0.71 (0.43-1.19)	60	30	1.04 (0.58-1.84)	0.25
		T/T	23	19	0.63 (0.31-1.28)	22	14	0.81 (0.38-1.75)	
rs1800825	*CCL5*	T/T	169	112	1	138	84	1.09 (0.76-1.56)	
		C/T	11	6	1.21 (0.44-3.38)	9	4	1.49 (0.45-4.96)	0.88
		C/C	0	0	---	1	0	---	

Table 13: Genotype distributions among different age groups in asthma cases and controls.

SNP	Genotypes	<18 years			18-40years			>40 years			p-value
		case	control	OR(95%CI)	case	control	OR(95%CI)	case	Control	OR(95%CI)	
rs1800871	C/C	12	24	1	61	31	3.94 (1.74-8.91)	55	15	7.33 (2.99-18.00)	0.76
	C/T	12	27	0.89 (0.34-2.35)	66	53	2.49 (1.14-5.44)	67	20	6.70 (2.85-15.74)	
	T/T	2	10	0.40 (0.08-2.12)	29	23	2.52 (1.04-6.10)	24	6	8.00 (2.58-24.81)	
rs1800896	A/A	13	33	1	82	67	3.11 (1.51-6.37)	80	19	10.69 (4.74-24.12)	0.0067
	A/G	13	21	1.57 (0.61-4.04)	57	35	4.13 (1.92-8.91)	50	18	7.05 (3.05-16.30)	
	G/G	0	7	0	16	2	20.31 (4.08-100.99)	14	2	17.77 (3.54-89.31)	
rs1295685	C/C	17	32	1	79	51	2.9 (1.4-5.79)	64	16	7.5(3.3-16)	0.87
	C/T	6	21	0.54 (0.18-1.59)	64	44	2.74 (1.36-5.53)	63	17	6.98 (3.15-15.46)	
	T/T	3	8	0.71 (0.17-3.01)	14	8	3.29 (1.15-9.41)	18	6	5.65 (1.89-16.89)	
rs1800925	C/C	19	39	1	92	68	2.78 (1.48-5.22)	86	27	6.54 (3.25-13.14)	0.66
	C/T	5	19	0.5(0.17-1.6)	54	34	3.2(1.62-6.5)	48	11	8.9(3.81-21)	
	T/T	2	3	1.3(0.21-8.8)	10	2	10.2(2.04-51)	9	1	18.4(2-156)	

continued

Table 13 Page 2

SNP	Genotypes	<18 years			18-40years			>40 years			p-value
		case	control	OR(95%CI)	case	control	OR(95%CI)	case	Control	OR(95%CI)	
rs20541	C/C	18	35	1	81	52	3.03 (1.56-5.90)	69	16	8.39 (3.82-18.42)	0.92
	C/T	5	19	0.51 (0.16-1.60)	61	45	2.64 (1.33-5.24)	55	19	5.63 (2.60-12.17)	
	T/T	3	7	0.83 (0.19-3.61)	13	9	2.81 (1.01-7.81)	18	6	5.83 (1.97-17.26)	
rs1946518	G/G	9	22	1	70	53	3.23 (1.37-7.58)	65	25	6.36 (2.58-15.67)	0.67
	G/T	14	26	1.32 (0.48-3.62)	62	37	4.10 (1.71-9.84)	58	10	14.18 (5.08-39.54)	
	T/T	3	11	0.67 (0.15-2.97)	24	13	4.51 (1.61-12.62)	18	4	11.00 (2.90-41.69)	
	A/A	20	38	1	101	71	2.70 (1.45-5.03)	88	25	6.69 (3.32-13.47)	
rs1801275	A/G	6	22	0.52 (0.18-1.48)	49	26	3.58 (1.74-7.36)	49	12	7.76 (3.38-17.82)	0.5
	G/G	0	1	0	7	7	1.90 (0.58-6.18)	8	2	7.60 (1.47-39.23)	
	A/A	18	36	1	104	66	3.15 (1.65-6.00)	97	23	8.43 (4.08-17.43)	
rs2280091	A/G	6	18	0.67 (0.23-1.97)	44	28	3.14 (1.50-6.57)	40	13	6.15 (2.65-14.31)	0.94
	G/G	1	5	0.40 (0.04-3.68)	4	9	0.89 (0.24-3.28)	4	3	2.67 (0.54-13.21)	
	C/C	8	33	1	70	51	5.66 (2.41-13.28)	75	23	13.45 (5.45-33.17)	
rs2682826	C/T	14	26	2.22 (0.81-6.09)	70	44	6.56 (2.78-15.50)	63	12	21.66 (8.06-58.21)	0.13
	T/T	4	2	8.25 (1.28-53.26)	15	9	6.87 (2.22-21.31)	6	4	6.19 (1.41-27.24)	

Table 13 Page 3

SNP	Genotypes	<18 years			18-40years			>40 years			p-value
		case	control	OR(95%CI)	case	control	OR(95%CI)	case	Control	OR(95%CI)	
rs3894194	C/C	5	19	1	44	25	6.69 (2.22-20.11)	34	11	11.75 (3.55-38.88)	
	C/T	10	28	1.36 (0.40-4.60)	78	50	5.93 (2.08-16.89)	75	18	15.83 (5.21-48.11)	0.33
	T/T	10	13	2.92 (0.81-10.56)	35	28	4.75 (1.58-14.32)	32	10	12.16 (3.61-40.96)	
rs8079416	C/C	5	18	1	46	24	6.90 (2.28-20.87)	36	12	10.80 (3.30-35.39)	
	C/T	10	29	1.24 (0.37-4.22)	78	50	5.62 (1.96-16.09)	76	17	16.09 (5.24-49.41)	0.22
	T/T	10	14	2.57 (0.71-9.26)	32	29	3.97 (1.31-12.07)	31	10	11.16 (3.29-37.82)	
rs11650680	C/C	17	38	1	101	65	3.47 (1.81-6.66)	79	25	7.06 (3.41-14.62)	
	C/T	8	19	0.94 (0.34-2.57)	52	35	3.32 (1.63-6.79)	53	12	9.87 (4.23-23.06)	0.77
	T/T	1	4	0.56 (0.06-5.38)	4	4	2.24 (0.50-10.01)	12	2	13.41 (2.70-66.60)	
rs17809012	A/A	10	21	1	32	35	1.92 (0.79-4.69)	31	10	6.51 (2.31-18.36)	
	A/G	10	23	0.91 (0.32-2.63)	78	51	3.21 (1.40-7.38)	76	22	7.25 (2.98-17.67)	0.33
	G/G	5	17	0.62 (0.18-2.15)	46	18	5.37 (2.12-13.60)	36	7	10.80 (3.57-32.63)	
rs4523	T/T	8	14	1	38	34	1.96 (0.73-5.23)	43	11	6.84 (2.29-20.39)	
	C/T	11	31	0.62 (0.21-1.88)	80	43	3.26 (1.27-8.37)	69	20	6.04 (2.22-16.43)	0.44
	C/C	7	13	0.94 (0.27-3.34)	35	30	2.04 (0.75-5.53)	31	10	5.42 (1.76-16.69)	

Table 13 Page 4

SNP	Genotypes	<18 years			18-40years			>40 years			p-value
		case	control	OR(95%CI)	case	control	OR(95%CI)	case	Control	OR(95%CI)	
rs528557	C/C	6	18	1	59	40	4.42 (1.62-12.12)	56	14	12.00 (4.02-35.83)	0.86
	C/G	15	31	1.45 (0.48-4.41)	59	49	3.61 (1.33-9.81)	55	16	10.31 (3.51-30.33)	
	G/G	5	9	1.67 (0.40-6.97)	26	15	5.20 (1.69-15.96)	23	7	9.86 (2.82-34.50)	
rs1342326	T/T	15	43	1	117	72	4.66 (2.41-8.99)	104	28	10.65 (5.18-21.89)	0.51
	G/T	9	15	1.72 (0.62-4.74)	31	32	2.78 (1.29-5.98)	38	12	9.08 (3.78-21.79)	
	G/G	2	2	2.87 (0.37-22.18)	7	3	6.69 (1.53-29.23)	5	1	14.33 (1.55-132.77)	
rs1800779	A/A	18	43	1	96	67	3.42 (1.82-6.44)	95	29	7.83 (3.93-15.60)	0.92
	A/G	7	16	1.05 (0.37-2.97)	51	34	3.58 (1.78-7.22)	41	11	8.90 (3.75-21.12)	
	G/G	1	1	2.39 (0.14-40.32)	8	6	3.19 (0.97-10.50)	8	1	19.11 (2.23-164.14)	
rs1805011	A/A	26	61	1	137	97	3.31 (1.96-5.62)	126	39	7.58 (4.23-13.58)	0.42
	A/C	0	0	---	16	9	4.17 (1.63-10.64)	17	2	19.94 (4.29-92.60)	
	C/C	0	0	---	1	1	2.35 (0.14-38.95)	2	0	---	

Table 13 Page 5

SNP	Genotypes	<18 years			18-40years			>40 years			p-value
		case	control	OR(95%CI)	case	control	OR(95%CI)	case	Control	OR(95%CI)	
	T/T	8	17	1	43	32	2.86 (1.10-7.43)	45	7	13.66 (4.29-43.48)	
rs2583476	C/T	9	28	0.68 (0.22-2.11)	74	49	3.21 (1.29-8.01)	74	18	8.74 (3.26-23.41)	0.77
	C/C	9	16	1.20 (0.37-3.86)	38	25	3.23 (1.21-8.61)	26	16	3.45 (1.21-9.83)	
	G/G	13	42	1	104	70	4.80 (2.40-9.59)	88	28	10.15 (4.78-21.57)	
rs543749	G/T	11	14	2.54 (0.93-6.94)	46	28	5.31 (2.43-11.57)	45	9	16.15 (6.26-41.70)	0.67
	T/T	2	3	2.15 (0.32-14.32)	4	3	4.31 (0.85-21.79)	8	2	12.92 (2.43-68.63)	
	G/G	20	53	1	129	87	3.93 (2.20-7.03)	127	36	9.35 (4.96-17.62)	
rs740347	C/G	4	7	1.51 (0.40-5.74)	23	13	4.69 (2.00-11.00)	15	3	13.25 (3.46-50.71)	0.42
	C/C	1	1	2.65 (0.16-44.42)	1	3	0.88 (0.09-9.00)	3	0	---	
	G/G	9	25	1	51	30	4.72 (1.95-11.45)	37	15	6.85 (2.60-18.07)	
rs1042713	A/G	15	29	1.44 (0.54-3.85)	81	48	4.69 (2.02-10.87)	77	16	13.37 (5.26-33.98)	0.52
	A/A	2	6	0.93 (0.16-5.45)	24	26	2.56 (1.00-6.58)	30	8	10.42 (3.50-30.99)	
	G/G	16	37	1	105	69	3.52 (1.82-6.81)	103	25	9.53 (4.59-19.80)	
rs1131882	A/G	10	19	1.22 (0.46-3.19)	47	30	3.62 (1.72-7.62)	38	9	9.76 (3.84-24.84)	0.66
	A/A	0	5	0	4	5	1.85 (0.44-7.81)	3	4	1.73 (0.35-8.66)	

Table 13 Page 6

SNP	Genotypes	<18 years			18-40years			>40 years			p-value
		case	control	OR(95%CI)	case	control	OR(95%CI)	case	Control	OR(95%CI)	
rs1799983	G/G	16	36	1	106	67	3.5(1.8-6.91)	100	28	8.(3.9-16.55)	0.97
	G/T	8	22	0.82 (0.30-2.23)	46	33	3.14 (1.50-6.57)	38	10	8.55 (3.43-21.29)	
	T/T	2	3	1.50 (0.23-9.87)	5	4	2.81 (0.67-11.88)	5	1	11.25 (1.21-104.24)	
rs1800469	C/C	8	23	1	78	46	4.87 (2.02-11.79)	59	16	10.6(4-28.13)	0.098
	C/T	15	25	1.72 (0.62-4.82)	52	45	3.32 (1.35-8.16)	67	14	13.76 (5.12-37.01)	
	T/T	3	11	0.78 (0.17-3.55)	26	13	5.75 (2.02-16.34)	16	9	5.11 (1.62-16.08)	
rs1800825	T/T	24	59	1	150	101	3.6(2.13-6.2)	133	36	9.08 (4.9-16)	0.96
	C/T	1	3	0.82 (0.08-8.28)	7	4	4.30 (1.15-16.06)	12	3	9.83 (2.55-37.98)	
	C/C	1	0	---	0	0	---	0	0	---	

4.5 GENOTYPING FOR TNF- A -308 G/A POLYMORPHISM

The genotype distribution of *TNF- α* -308 G/A polymorphism were not in HWE within each group that is cases or controls. The genotypes and alleles frequencies of *TNF- α* -308 G/A polymorphism in asthma cases and control subjects are shown in Table 14 and 15. We did not find any significant difference in the prevalence of this polymorphism between cases and control subjects of studied Pakistani population.

TNF- *α* is a potential candidate gene among many genes involved in the induction of airway inflammation. Given the biological regulation of *TNF-α* and its role in the inflammatory process, it is perhaps surprising that the genetic influences on cytokine production have much influence on disease processes and their outcome. The associations between *TNF-α* genotype and disease are not absolute as suggested by different conflicting studies (Witte *et al.,* 2002; Randolph *et al.,* 2005). Nevertheless, it is clear that the genetic regulation of *TNF-α* at sites of inflammation is important. Under circumstances where the release of *TNF-α* has triggered the genetically endowed capacity for greater *TNF-α* production leads to more severe inflammatory reactions.

Previous studies have shown an association between the *TNF- α* -308 G/A polymorphism and asthma (Gupta *et al.,* 2005; Louis *et al.,* 2000). In present study lack of *TNF- α* -308 G/A polymorphism association with asthma suggests that -308 G/A allele could not be considered as a genetic factor for susceptibility to asthma in our studied population. These results can be re-analyzed by increasing number of

cases and controls and the results in future studies and its role as a disease marker could be confirmed.

Table 14: Genotypes, χ^2 and Odds Ratio (OR) of TNF α-308 in asthma cases and controls.

Genotype	Asthma patients n=329	Controls n=151	χ^2	OR (95% CI)	p-value
GG	88(27%)	39(26%)	0.05	1.049 (0.68-1.63)	0.823
GA	241(73%)	111(73%)	0	0.987 (0.64-1.53)	1
AA	0(0%)	1 (0.006%)	--	-- (--)	--

Table 15: Alleles, χ^2 and Odds Ratio (OR) of cases (Asthma) and controls for TNF alpha-308.

Alleles	Asthma Cases n=329	controls n=151	χ^2	OR (95% CI)	p-value
G	417	189	0.06	1.03 (0.78-1.37)	0.806
A	241	113	0.06	0.967 (0.73-1.28)	0.806

4.6 GENOTYPING FOR ACE I/DPOLYMORPHISM

The genotype distribution of *ACE* I/D polymorphism were in HWE within each group of cases or controls. The frequency of genotypes and alleles of *ACE*I/D in the asthma cases and controls are presented in Table 16 and 17. This marker has shown strong association with disease in our population ($p \leq 0.0001$).Our genotyping results indicate that homozygous insertion (II) significantly increased the risk of asthma (OR=3.38, 95% CI=2.35-4.84) as compared to deletion which The heterozygous insertion/deletion (ID) seemed to have a protective role with regards to asthma susceptibility (OR=0.43, 95%CI=0.33-0.57 and $p \leq 0.0001$).

Followed by genotypes, ACE I/D alleles were also analyzed separately in cases and controls and checked for their association with asthma in studied Pakistani population. As presented in Table 17, these results have shown that I allele is strongly associated with asthma risk in studied population (OR=1.4, 95% CI=1.15-1.7, p=0.0007) whereas deletion allele is showing association towards asthma protection (OR=0.71, 95% CI=0.59-0.87, p=0.0007).

The insertion/deletion (I/D) polymorphisms of ACE gene have also been implicated in susceptibility to asthma as it affects serum ACE levels, but many earlier studies have reported inconclusive results. Angiotensin converting enzyme DD genotype has been reported as more prevalent in asthmatics (Benessiano *et al.*, 1997; Gao *et al.*, 2000) and has also stated that *ACE* DD genotype is related with severe disease presentation (Eryuksel *et al.*, 2009; Gao *et al.*, 2000). However, others did not find association between *ACE* I/D genotypes and asthma or asthma severity (Chagani *et al.*, 1999; Lee *et al.*, 2000; Nakahama *et al.*, 1999).

Table 16: Genotype frequencies, χ^2 and Odds Ratio (OR) of ACE I/D polymorphism in asthma cases and controls.

Genotype	Asthma patients	Controls	χ^2	OR (95% CI)	p-value
	n=333	n=521			
II	99(30%)	58 (11%)	46.83	3.38 (2.35-4.84)	<0.0001
ID	140 (42%)	326(63%)	34.54	0.43 (0.33-0.57)	<0.0001
DD	94(28%)	137(26%)	0.38	1.11 (0.81-1.5)	0.54

Table 17: Allele frequencies, χ^2 and Odds Ratio (OR) of ACEI/D in asthma cases and controls.

Alleles	Asthma Cases	controls	χ^2	OR	p-value
	n=333	n=521		(95% CI)	
I	338(51%)	442(42%)	11.37	1.40 (1.15-1.70)	0.0007
D	328(49%)	600(58%)	11.37	0.71 (0.59-0.87)	0.0007

A recent meta-analysis has suggested that I/D polymorphism of the ACE gene would be a risk factor for asthma (Zhang *et al.,* 2011). Results of this meta-analysis indicated that DD homozygote carriers had almost 59 per cent increased risk of asthma and this risk is more evident in Asians, especially in children. In contrast, a study conducted on adult Turkish population (Yildiz *et al.,* 2004; Urhan *et al.,* 2005) reported that ACE gene I/D polymorphisms are not an important determinant of asthma susceptibility. Our study is the first from Pakistan exploring relationship between *ACE* I/D genotype and asthma risk.

Different results from the literature about *ACE* I/D polymorphisms may be explained by the genetic heterogeneity and multifactorial etiology of asthma. Multicentre studies with a large number of patients are required to confirm these results. In the present study, it was concluded that homozygous II gene polymorphism is a risk factor for asthma in studied Pakistani population.

4.7 HLA TYPING OF CLASS II ALLELES IN ASTHMA

Twenty-five HLA class II alleles were tested in relation to asthma incidence using available subjects for each allele results are summarized in tables 18 and 19. Of these 25 alleles, 14 alleles were of HLA Class II locus DRB1 and 11 of locus DQB1. Of DRB1 locus, HLA DRB1*0701 allele was significantly associated ($p=0.01$) towards an increased risk of asthma (OR=1.586; 95% CI=1.150-2.186). Several studies on diverse world populations have reported association of DRB1*07 with asthma/disease. A study on 109 patients of allergy in United

Kingdom has shown that DRB1*07 is seen to be associated with increased risk of asthma (Jeal *et al.,* 2003). In another study on venome, sensitive cases has reported DRB1*07 association with allergy (Faux *et al.,* 1997). While in a study on Iranian cases of pulmonary disease, DRB1*07 was reported to be positively associated with increased risk of disease (Amirzargar *et al.,* 2004). All these studies are in parallel with ours showing DRB1*07 as a strong disease marker. In comparison one study on Slovak population reported lack of association of DRB1*07 with disease (Dzurilla *et al.,* 2013). The difference may be due to different genetic makeup and different environmental agents responsible for asthma in studies showing disease association with DRB1*07.

The DQB1* allele results are shown in table19. Out of the 11 alleles of the DQB1* locusDQB1*03032 allele was associated with an increased risk of asthma in our studied Pakistani population (OR=2.42; 95% CI=1.34-4.35; p=0.02). While two alleles; DQB1*06 and DQB1*0602 showed trend towards protection from asthma (Table 9). According to our findings DQB1*06 allele carriers (OR=**0.39; 95%CI=0.22-0.70; p≤0.0001) and** DQB1*0602 allele carriers (OR=**0.27**; **95%CI= 0.10-0.71; p≤0.0001)** significantly decrease the risk of disease. In contrast to our findings, a study of 311 Caucasian Canadian allergy patients and 226 controls reported DBB1*06 allele to be associated with disease susceptibility (Madore *et al.,* 2013). In another study on 109 asthma patients of Slovak population, it was seen that DQB1*03 and DQB1*06 lack association with disease (Dzurilla *et al.,* 2013).

The other 21 HLA class II alleles of interest lacked significance in association with asthma incidence in our population. In present study, we have found that the frequency of the HLA class II alleles DQB1*03032 was significantly high among asthma patients as compared to ethnically matched healthy control subjects while the frequency of DQB1*06 and DQB1*0602 significantly decreased.

4.7.1 Haplotype Analysis Of HLA Class II

The two locus haplotype for the HLA loci DRB1 and DQB1 are made by Arlequin. In this, 115 haplotypes were seen in cases and 128 haplotypes were observed in controls. In these 34 haplotypes have frequencies of more than 2 per cent in each group and these > 2 % haplotypes are shown in table20.

In the table 20 it is shown that some of the haplotypes such as DRB1*01-DQB1*05, DRB1*11-DQB1*0601, DRB1*12-DQB1*0301/0304, DRB1*13-DQB1*06, DRB1*1302-DQB1*06, DRB1*15-DQB1*06 have shown significant p values but in the analysis their OR and 95% CI did not coincide with those p values. Therefore, these were showing wrong associations. When we look at the haplotype frequencies in both cases and controls it was also confirmed that there was not much difference between both subjects. The haplotypes DRB1*13-DQB1*02, DRB1*15-DQB1*05 and DRB1*15-DQB1*0603 have shown significant p values but the LD is negative so here it is not taken into consideration

as they are not in LD. Therefore, these haplotypes are not showing the right significance.

The haplotypes DRB1*0701-DQB1*06, DRB1*14-DQB1*05 and DRB1*15-DQB1*0602 were present only in controls and not found in cases and these haplotype were seen to be significantly associated with protection in the Pakistani population. DRB1*0701-DQB1*03032was the haplotype that is present in both cases and controls with significant p value with OR and 95% CI. All these have shown its association with protection in Pakistani population. These all haplotypes were following the linkage disequilibrium (LD) and is significantly associated with asthma protection in Pakistani population (Table20).

Table 18: DRB1* alleles frequencies, F test, Odds Ratio (OR) and p values in cases and controls.

DRB Alleles	Freq in Cases	Freq in Controls	F test	P	P corrected	OR (95% CI)
DRB1*01	0.03	0.04	0.66	0.42	5.84	0.79 (0.45-1.39)
DRB1*03	0.19	0.20	0.06	0.80	11.24	0.97 (0.74-1.26)
DRB1*04/1122	0.07	0.07	0.07	0.79	11.06	1.06 (0.70-1.59)
DRB1*0701	0.15	0.10	8.06	**0.01**	**0.07**	**1.59 (1.15-2.19)**
DRB1*08	0.13	0.01	0.31	0.58	8.11	1.32 (0.49-3.54)
DRB1*09	0.02	0.01	1.21	0.27	3.82	1.57 (0.69-3.53)
DRB1*1001	0.05	0.04	1.89	0.17	2.38	1.43 (0.86-2.38)
DRB1*11	0.08	0.10	1.95	0.16	2.28	0.77 (0.53-1.11)
DRB1*12	0.01	0.02	0.08	0.77	10.84	0.88 (0.36-2.16)
DRB1*13	0.08	0.08	0.10	0.75	10.47	0.94 (0.64-1.38)
DRB1*1302	0.02	0.02	0.32	0.58	8.05	1.23 (0.59-2.58)
DRB1*14	0.04	0.05	3.06	0.08	1.12	0.63 (0.37-1.06)
DRB1*15	0.22	0.24	0.51	0.48	6.68	0.91 (0.71-1.17)
DRB1*16	0.01	0.02	0.08	0.77	10.84	0.88 (0.36-2.16)

Table 19: DQB1* alleles frequencies, F test, Odds Ratio (OR) and p values in cases and controls.

DQB Alleles	Freq in Cases	Freq in Controls	F test	P	P corrected	OR (95% CI)
DQB1*02	0.26	0.26	0.00	0.96	10.51	1.01 (0.79-1.28)
DQB1*0301/0304	0.14	0.12	0.28	0.60	6.53	1.09 (0.79-1.49)
DQB1*0302	0.04	0.03	1.65	0.20	2.19	1.45 (0.82-2.54)
DQB1*03032	0.05	0.02	9.22	**0.00**	**0.02**	**2.42 (1.34-4.35)**
DQB1*0305	0.01	0.00	1.23	0.27	2.95	2.21 (0.53-9.26)
DQB1*04	0.02	0.01	3.26	0.07	0.78	2.44 (0.90-6.64)
DQB1*05	0.20	0.21	0.01	0.91	9.98	0.98 (0.76-1.28)
DQB1*06	0.03	0.06	10.73	**0.00**	**0.01**	**0.39 (0.22-0.70)**
DQB1*0601	0.15	0.14	0.16	0.69	7.56	1.06 (0.79-1.43)
DQB1*0602	0.01	0.03	8.15	**0.00**	**0.04**	**0.27 (0.10-0.71)**
DQB1*0603	0.91	0.11	0.95	0.33	3.62	0.84 (0.59-1.20)

Table 20: DQB1* alleles frequencies, F test, Odds Ratio (OR) and p values in cases and controls.

Haplotype	Patients		Controls		ANOVA	Odds Ratio	
	HF (%)	LD(x100)	HF (%)	LD(x100)	F-test	(95%CI)	p-value
01 05	2.64	1.97	4.14	3.27	3.886	0.541(0.291-1.006)	0.049
03 02	16.23	11.24	17.27	12.05	0.231	0.934(0.706-1.235)	0.631
03 0301/0304	1.16	-1.40	0.25	-2.11	2.968	2.049(0.891-4.714)	0.085
03 03032	0.19	-0.86	0.28	-0.19	0.409	1.393(0.502-3.862)	0.522
03 05	0.86	-3.03	1.15	-3.05	0.149		0.537
03 0603	0.19	-1.54	0.55	-1.59	1.368	0.656(0.322-1.336)	0.242
04 02	.....	.....	0.28	-0.83	12.572		0
04 0301/0304	1.15	0.18	2.13	2.00	0.844	0.650(0.258-1.639)	0.358
04 0302	3.63	3.32	.....	.....	26.341		0

continued

Table 20 Page 2

Haplotype	Patients		Controls		ANOVA	Odds Ratio	
	HF (%)	LD(x100)	HF (%)	LD(x100)	F-test	(95%CI)	p-value
0701 02	8.17	4.25	5.96	3.35	3.469	1.447(0.979-2.138)	0.063
0701 0301/0304	0.83	-1.18	0.13	-1.05	1.261	1.682(0.672-4.207)	0.261
0701 03032	4.43	3.61	1.73	1.49	7.333	**2.402(1.249-4.621)**	**0.007**
0701 06	…….	……..	1.39	0.74	4.705	**0.218(0.048-0.989)**	**0.03**
0701 0601	……..	……	0.13	-1.29	5.037	0.134(0.017-1.057)	0.025
1001 05	4.60	3.53	……	……	35.527		0
11 0301/0304	7.41	6.33	…….	……	58.745		0
11 0601	…….	…….	0.50	-0.94	5.037	0.134(0.017-1.057)	0.025
12 0301/0304	1.16	0.98	……..	……	8.584		0.003
13 02	……	……	0.13	-2.06	12.47	0.066(0.009-0.504)	0

Table 20 Page 3

Haplotype	Patients		Controls		ANOVA	Odds Ratio	
	HF (%)	LD(x100)	HF (%)	LD(x100)	F-test	(95%CI)	p-value
13 0301/0304	0.66	-0.39	…..	…….	12.322		0
13 05	0.63	-0.98	……	……	7.346		0.007
13 06	……	…….	1.24	0.71	5.037	0.134(0.017-1.057)	0.025
13 0601	…….	……..	0.17	-1.02	7.487		0.006
13 0603	6.26	5.55	6.51	5.62	0.208	0.905(0.589-1.39)	0.649
1302 06	…….	……...	1.23	1.11	5.037	0.134(0.017-1.057)	0.025
14 05	…….	………	4.87	3.77	27.292	**0.061(0.015-0.254)**	**0**
15 02	0.17	-5.55	0.39	-5.75	0.009	1.029(0.577-1.833)	0.924
15 0301/0304	…..	…….	0.25	-2.52	16.883		0
15 05	5.63	1.18	4.09	-0.84	4.735	1.666(1.047-2.649)	0.03

Table 20 Page 4

Haplotype	Patients		Controls		ANOVA	Odds Ratio	
	HF (%)	LD(x100)	HF (%)	LD(x100)	F-test	(95%CI)	p-value
15 06	……..	……	0.92	-0.58	9.175		0.003
15 0601	13.97	10.64	12.73	9.40	0.163	1.066(0.783-1.451)	0.687
15 0602	……..	……...	2.92	2.18	10.579	**0.170(0.05-0.571)**	**0.001**
15 0603	0.99	-1.00	2.12	-0.39	6.545	0.347(0.149-0.811)	0.011
16 05	0.97	0.70	1.13	0.81	0.03	0.910(0.314-2.637)	0.862

HF (Haplotype frequency), LD (linkage Disequilibrium), ANOVA (Analysis of Variance)

4.7.2 META-ANALYSIS OF HLA DRB1*0701 AND HLA DQB1*06 TYPING AND ASTHMA

The results of HLA DRB1*0701 and HLA DQB1*06 meta-analysis are given in Table21 and 22 and interpretation by forest plot is shown in Figure 13 and14. Meta-analysis was done using data of HLA DRB1*0701 typing of 5 studies of UK, American, Irani, Slovak and Pakistani population (594 patients and 1070 controls) and HLA DQB1*06 typing based on 3 studies of Caucasians, Slovak and Pakistani population (723 patients and 768 controls). In forest plots each square represents the OR point estimate and its size is proportional to the weight of the study. The diamond shape represents the overall summary estimate, with confidence interval given by its width.

A significant association (p=0.00) between the HLA-DRB1*07 and asthma was observed in over all analysis with an OR of 1.607 (95%CI of 1.299-1.987) in considering Fixed Model while in random model this significance was retained (p=0.001) with an OR of 1.633 (95%CI of 1.212-2.202). The Meta analysis of DRB1*07 including our study proves the authenticity of DRB1*07 our significance results in comparison with other world populations (Jeal *et al.,* 2003; Faux *et al.,* 1997; Amirzargar *et al.,* 2004). According to forest plot the study on different populations favors our study (Figure 14). The present study was showing the same trend of association of DRB1*07 with risk of asthma as in other world populations considered as shown in figure 14.

In case of HLA-DQB1*06 no significant associations was found in combined analysis in either of the models (Table 22). The forest plot of the study has also shown contradictory results (Figure 13). For Meta analysis of HLA DQB1*06 we did not find enough studies where this allele is reported for association with asthma. Only two populations (Caucasians and Slovak) were found having DQB1*06 association with asthma. Therefore, due to less number of studies the significance of association might have been lost. However, we did find association in our population in the present study and it is possible that this association is population specific as many studies have shown population specific associations. For example, in a previous study on HCV in Pakistani population there was an association of disease with DRB1*07 and DQB1*02 (Ali *et al.,* 2010). In another study on Pakistani population rheumatic heart disease was reported to be associated with DRB1*07 (Rehman *et al.,* 2007). Therefore, it is possible to observe population specific associations as well as those shown by some previous studies (Ali *et al.,* 2010; Rehman *et al.,* 2007). The present association of asthma with DQB1*06 can help clinicians for further management of the disease.

Table 21: Meta-analysis of case-control studies of HLA DRB1*07.

Model	Total Studies	OR (95%CI)	Z-value	p-value
	5			
Fixed		1.607(1.299-1.987)	4.378	0.000
Random		1.633(1.212-2.202)	3.219	0.001

Table 22: Meta-analysis of case-control studies of DQB1*06.

Model	Total Studies	OR (95%CI)	Z-value	p-value
	3			
Fixed		0.925(0.68-1.26)	-0.492	0.623
Random		0.997(0.368-2.699)	-0.006	0.995

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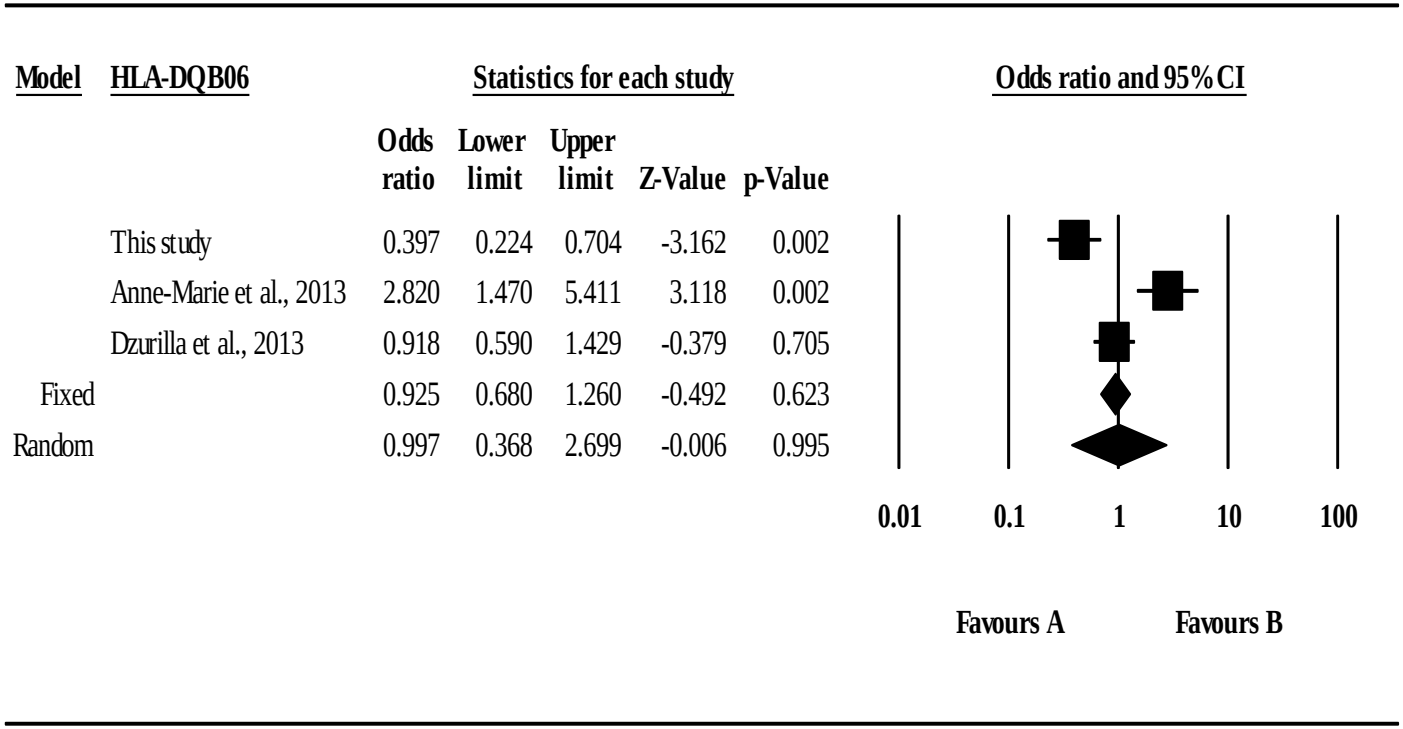

Figure 13: Forest plot of the DQB1*06 and asthma in over all analysis.

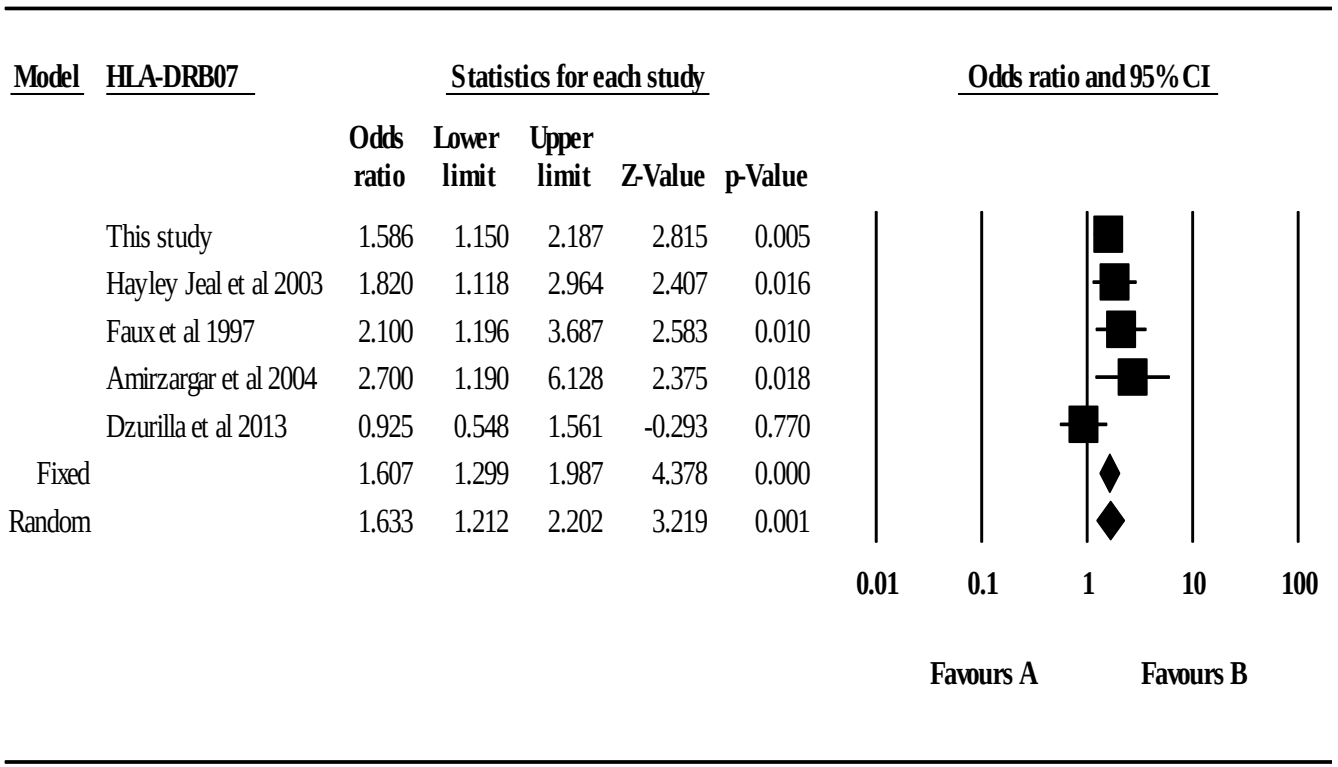

Figure 14: Forest plot of the *DRB1*07* and asthma in over all analysis.

SUMMARY

Asthma is a chronic inflammatory disorder of lungs and airways. It is characterized by specific symptoms for example bronchial arrest, narrowing of airways, wheezing and cough. In spite of the development the affected individuals number is increasing day by day all around the world. About 300 million people worldwide and almost 20 million people in Pakistan are the victims. During asthmatic attack special types of cells such as eosinophils and Th2 cells accumulate in airways that narrow the airways and patient feel difficulty in breathing.

Asthma is an interactive disease and is caused by combination of environmental and genetic factors. A number of genes are found to be associated with asthma in different studies. Many single nucleotide polymorphisms (SNPs) are found in these genes and their association with different diseases has been established. Many of the SNPs of *ADAM33, IL10, IL13* and *TBXA2R* are found to be associated with asthma and other respiratory disorders and their association is confirmed by genome wide association studies and linkage analysis in different population. But Genetic screening of Pakistani population for association between these and some other reported genetic variants and asthma has yet not been established. Therefore the proposed study was designed to screen the local population to check the association under following objectives:

The purpose of the present study is the genetic analysis of asthmatic Pakistani population and study of candidate genes and polymorphisms associated with asthma in Pakistani population. For this purpose

854 samples of healthy and asthma patients were genotyped by using Sequence specific PCR, iPLEX and Taqman assay. HLA class II was typed by using sequence specific PCR. For SNP analysis 34 SNPs of 28 different genes were genotyped and ACE I/D polymorphism was also genotyped. To check the association of SNPs with asthma with specific gender and age groups statistical analysis was performed. These results clearly shows that there is a strong association between asthma susceptibility, and *IL10*(rs1800896) and *IL13* (1800925) minor alleles; however, an association with protection was found between *ADAM33*(rs2280091) and asthma in Pakistani study population. For *FCER1B* gene, rs2583476 has shown a significant genotype distribution in males. In our sample population of male gender TT genotype is highly prevalent in asthma cases as compared to healthy controls. In *ORMDL3* rs11650680 has shown the significant genotype distribution in females. In our sample population of female gender CT genotype is highly prevalent in asthma cases as compared to healthy controls. In rs1800896 (*IL10*) significant association has been shown in age group 18-40 years and more than 40 years of age. For *ACE* I/D polymorphism the II genotype is a risk factor for asthma in Pakistani population.

HLA DRB1*0701 and HLA DQB1*03032 was significantly associated with an increased risk of asthma in current study. DQB1*06 and DQB1*0602 were reported to be associated with protection of asthma in present study population. The haplotypes DRB1*0701-DQB1*06, DRB1*14-DQB1*05, DRB1*0701-DQB1*03032 and DRB1*15-DQB1*0602 were seen to be significantly associated with protection in the Pakistani population.

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APPENDICES

Appendix I

Table 1: List of 20 SNPs done by iPLEX and their amplification primers sequence with amplicon lengths

SNP_ID	Amplification Primer sequence	Amplicon Length (bp)
rs1800896	ACGTTGGATGGACAACACTACTAAGGCTTC	94
rs1295685	ACGTTGGATGAATGAGTGTGTTTGTCACCG	100
rs1801275	ACGTTGGATGACCCTGCTCCACCGCATGTA	108
rs1042713	ACGTTGGATGAGCGCCTTCTTGCTGGCA	97
rs1799983	ACGTTGGATGACCTCAAGGACCAGCTCGG	96
rs1800925	ACGTTGGATGGGGTTTCTGGAGGACTTCTA	80
rs1131882	ACGTTGGATGTCATGGCAGGCGGGTTTCG	85
rs543749	ACGTTGGATGTGCCACACAGCTTGCAGCC	84
rs2280091	ACGTTGGATGCTGTCCAGTGGCTGTGGG	97
rs2289278	ACGTTGGATGTAGGCGGCAAAGTTTACGAG	100
rs1800825	ACGTTGGATGGCATTGGCCGGTATCATAAG	100
rs1800469	ACGTTGGATGAGGGTGTCAGTGGGAGGAG	100
rs11650680	ACGTTGGATGAAAAGACTGTCAGAGAAGGC	98
rs1946518	ACGTTGGATGTGCTGTATCAGATGCAAGCC	100
rs8079416	ACGTTGGATGTGTCTGGATCACTCTGTTGG	100
rs3894194	ACGTTGGATGAAGTCGATGAGGCTGTCAAG	100
rs3771180	ACGTTGGATGGATTTGTGGCCAAATCTATG	91
rs2682826	ACGTTGGATGGCCATGTTCCAGTGGTTTCA	93
rs740347	ACGTTGGATGATTCACCCTTCCACAACAGC	89
rs17809012	ACGTTGGATGGCAAGAAAACCAAGGCCCTA	99

Appendix II

Table 2: List of 8 SNPs done by iPLEX and their amplification primers sequence with amplicon lengths

SNP_ID	Amplification Primer sequence	Amplicon Length (bp)
rs2583476	ACGTTGGATGGAACAATTACTGATGTTCAAC	93
rs1805011	ACGTTGGATGAGGAACAGGCTCTCTGTTAG	90
rs1800779	ACGTTGGATGACCAGATGCCCAGCTAGTG	89
rs1342326	ACGTTGGATGGAAGTATTTGGAGTCCAAAAG	93
rs1800871	ACGTTGGATGGACCCCTACCGTCTCTATTT	95
rs528557	ACGTTGGATGAGTCGGTAGCAACACCAGG	100
rs4523	ACGTTGGATGCTGGAACCAGATCCTGGAC	97
rs20541	ACGTTGGATGTGATGCTTTCGAAGTTTCAG	100

Table 3: List of 8 SNPs done by iPLEX and their extension primers sequence with their calls and masses

SNP_ID	EXT1_Seq	E2 Call	E2 Mass	EXT2_Seq
rs2583476	CCATGCGCCTTTATGTAC	T	5463.4	CCATGCGCCTTTATGTAT
rs1805011	CTTCCAGGAGGGAAGGGC	A	5571.7	CTTCCAGGAGGGAAGGGA
rs1800779	GGGGTTTGTAGTTCTGTGC	T	5878.8	GGGGTTTGTAGTTCTGTGA
rs1342326	TTTTCTCATGAAGACACCAG	T	6073.9	TTTTCTCATGAAGACACCAT
rs1800871	CCCTTGTACAGGTGATGTAAC	T	6459.1	CCCTTGTACAGGTGATGTAAT
rs528557	CCTGCTGCCTCTGCTCCCAGGC	C	6597.3	CCTGCTGCCTCTGCTCCCAGGG
rs4523	CACGGCGCGGCGGAACAGGATA	C	6793.4	CACGGCGCGGCGGAACAGGATG
rs20541	AAAGAAACTTTTTCGCGAGGGACC	T	7367.8	AAAGAAACTTTTTCGCGAGGGACA

Appendix III

Table 4: List of 20 SNPs done by iPLEX and their extension primers sequence with their calls and masses

SNP_ID	EXT1_Seq	E2 Call	E2 Mass	EXT2_Seq
rs1800896	CCTATCCCTACTTCCCCC	A	5304.3	CCTATCCCTACTTCCCCT
rs1295685	CCCTTGGCTCCAAGTGCC	T	5449.4	CCCTTGGCTCCAAGTGCT
rs1801275	tCCCCACCAGTGGCTATCA	G	5682.7	tCCCCACCAGTGGCTATCG
rs1042713	GGTCCGGCGCATGGCTTCC	A	5834.7	GGTCCGGCGCATGGCTTCT
rs1799983	caCTGCAGGCCCCAGATGAG	T	6109.9	caCTGCAGGCCCCAGATGAT
rs1800925	tCTTTTCCTGCTCTTCCCTCA	C	6199	tCTTTTCCTGCTCTTCCCTCG
rs1131882	TCCCCTTTGCAGGTCTTCATC	A	6337	TCCCCTTTGCAGGTCTTCATT
rs543749	GAGGATATGTTGTCCCCTAAG	T	6459.1	GAGGATATGTTGTCCCCTAAT
rs2280091	ttccGGGCGGCGTTCACCCCAC	A	6686.2	ttccGGGCGGCGTTCACCCCAT
rs2289278*	CTCCCCTTTCACTCAATTCTCAC	G	6803.5	CTCCCCTTTCACTCAATTCTCAG

rs1800825	CCGGAGGCTATTTCAGTTTTCTC	T	7034.5	CCGGAGGCTATTTCAGTTTTCTT
rs1800469	gattCCTCCTGACCCTTCCATCCC	T	7173.5	gattCCTCCTGACCCTTCCATCCT
rs11650680	aaaAAAACCAGCTCAAAATTCTCC	T	7304.7	aaaAAAACCAGCTCAAAATTCTCT
rs1946518	TGCAGAAAGTGTAAAAATTATTAC	T	7389.9	TGCAGAAAGTGTAAAAATTATTAA
rs8079416	aattGTTTTCCTGCCACCAATACAC	T	7573.8	aattGTTTTCCTGCCACCAATACAT
rs3894194	cCCCTGGCCAGACAGCTAAACCCTCA	C	7804.1	cCCCTGGCCAGACAGCTAAACCCTCG
rs3771180	ACATCAAGAATTCTTAGTACATGATG	A	7975.1	ACATCAAGAATTCTTAGTACATGATT
rs2682826	cccCTCTTGCCGACAAGGGCAACTCAC	T	8188.2	cccCTCTTGCCGACAAGGGCAACTCAT
rs740347	gcatGGTTGTTCAGATTATTTATGCTC	C	8284.4	gcatGGTTGTTCAGATTATTTATGCTG
rs17809012	gacgTTGGTTTCCTTGCTCCTTTCCCCC	A	8406.5	gacgTTGGTTTCCTTGCTCCTTTCCCCA

To Study the Role of Genetic Factors in the Development and Treatment of Allergy and Asthma in the Pakistani Population

S. No. ..... Date ..... Vaccine No. .....

Name:

.....

Age: ..... Sex: Male / Female Marital

Status.....

Address:

.....

.....

.....

.....

Tel. No. .....

Thank you for your participation in the above mentioned research programme. The purpose of our research is to identify the genetic factors that predispose Pakistani patients to allergy and asthma. Patients included in the study will be requested to donate a blood sample at the Allergy and Asthma Centre (AACP), Islamabad. The sample shall be taken with a sterile syringe. The sample shall be sent to the Institue of Biomedical and Genetic Engineering, Dr. A. Q. Khan Research Laboratories, Islamabad, for genetic analyses. The blood sample will not be utilized for any other research purpose. Strict confidentiality will be maintained and the patient will be kept informed with the details of the findings.

درج بالا تحقیقاتی مطالعے میں آپ کے تعاون کا شکریہ:
ہماری اس تحقیق کا مقصد ان موروثی عوامل کا پتہ لگانا ہے جو پاکستانی عوام میں الرجی یا دمہ کا باعث بنتے ہیں۔اس سلسلے میں الرجی اور دمہ کلینک اسلام آباد میں علاج کیلئے آنے والے الرجی اور دمہ کے مریضوں سے التماس ہے کہ وہ اپنے خون کا نمونہ جمع کروادیں۔
خون کا یہ نمونہ ایک تجربہ کار نرس جراثیم سے پاک سرنج میں حاصل کرے گی جس کو بعد ازاں جینیاتی تحقیق کیلئے کے آر ایل کے شعبہ بایومیڈیکل اور جینیٹک انجینئرنگ میں بھجوایا جائے گا۔

حاصل کئے گئے خون کے یہ نمونے صرف تحقیقاتی مقاصد کیلئے استعمال ہونگے۔
مریض کے کوائف کے بارے میں مکمل رازداری کا اہتمام کیا جائے گا۔
تحقیق کے نتیجے میں حاصل ہونے والے نتائج سے باخبر رکھا جائے گا۔

رضامندی:
میں اپنا خون صرف تحقیقاتی مقاصد کیلئے عطیہ کر رہا/رہی ہوں۔یہ کسی تجارتی مقصد کیلئے استعمال نہیں کیا جاسکتا۔

INFORMED CONSENT:
I am donating my blood for research purposes only and not for commercial use.

Signature:_____